08/22/01 → 12/15/93

6/12/93 - CIP (NOT RUN?)

**DOCKET NO: UPVG0003-103**
**PATENT APPLICATION**

**Serial No.: 09/935,100**
**Filed: August 22, 2001**

**AMENDMENTS TO THE CLAIMS:** DRC: α-VPR INHIBITED EXTRACELL; VPR IN VITRO; NOT IN VIVO

Please cancel claims 35, 39, 42 and 45.

This listing of claims will replace all prior versions, and listings, of claims in the (CRU-CRU) application:

**Listing of Claims** 32-34, 36-38, 40, 41, 43, 44, 46

Claims 1-31 **(Canceled)**

102/103 32. **(Previously presented)** A pharmaceutical composition comprising Ab: 32, 36, 37, 38, 40 MATH: 33, 34, 41, 43, 44, 46

a) anti-Vpr monoclonal antibodies; and

b) a pharmaceutically acceptable carrier.

5156949 col 14 33. **(Previously presented)** A method of treating an individual exposed to HIV by administering an effective amount of anti-Vpr antibodies. → SPECIFICITY? TETR?

34. **(Previously presented)** A method of treating an individual who has been infected with HIV comprising the step of administering to said individual a therapeutically effective amount of anti-Vpr antibodies.

35. **(Canceled)**

NM 36. **(Previously presented)** The pharmaceutical composition of claim 32 wherein the anti-Vpr antibodies bind to a fragment of (Vpr comprising amino acids 2-12) → NEW MATTER CEQAPEDQGPQ (2, 5, 10)

102/103 37. **(Previously presented)** A pharmaceutical composition comprising:

a) anti-Vpr antibodies that inhibit Vpr enhancement of HIV replication; and

b) a pharmaceutically acceptable carrier;

wherein the anti-Vpr antibodies are present in an amount effective to inhibit HIV replication in an HIV infected individual.

102/103 38. **(Previously presented)** The pharmaceutical composition of claim 37 wherein the anti-Vpr antibodies are monoclonal antibodies.



5/3/06
4/6/06

39. **(Canceled)**

N.M. 102/103 40. **(Previously presented)** The pharmaceutical composition of claim 37 wherein the composition is a sterile composition and the anti-Vpr antibodies bind to a fragment of Vpr comprising amino acids 2-12.

102/103 41. **(Previously presented)** The method of claim 33 wherein the anti-Vpr antibodies are monoclonal antibodies.

42. **(Canceled)**

102/103 NM 43. **(Previously presented)** The method of claim 33 wherein the anti-Vpr antibodies bind to a fragment of Vpr comprising amino acids 2-12.

102/103 44. **(Previously presented)** The method of claim 34 wherein the anti-Vpr antibodies are monoclonal antibodies.

45. **(Canceled)**

102/103 NM 46. **(Previously presented)** The method of claim 34 wherein the anti-Vpr antibodies bind to a fragment of Vpr comprising amino acids 2-12.

Cell specific envelope proteins are well known. A chimeric gene is designed which includes the portion of the *vpr* protein that binds to p24 together with a biological active protein which retains its activity when linked to the portion of *vpr*. Cells are co-transfected with a nucleic acid molecule that encodes the desired *env*, the chimeric gene, a nucleic acid molecule that encodes p24 or a nucleic acid molecule that encodes the full length *gag* precursor plus the HIV protease. Expression of these sequences will result in the proteins thus encoded being produced and assembly of the drug delivery particle. Noncoding RNA may also be provided for safety since the assembling particle will package RNA.

Biologically active proteins which can be used in fusion proteins include cytokines, lymphokines, structural proteins such as dystrophins, other therapeutic proteins and proteins which are useful as immune targets.

As an immunotherapeutic, the administration of *vpr* or an immunogenic fragment of *vpr*, particularly an inactive, i.e. non-functional, immunogenic fragment, provides a target against which an individuals immune system can mount an immune response which will recognize viral produced *vpr* and inactivate it. The *vpr* or fragment thereof is preferably eukaryotically produced. It is administered in a dose sufficient to evoke a protective immune response. Multiple doses may be administered. One having ordinary skill in the art can readily formulate an immunogenic composition that comprises *vpr* or fragment thereof. Adjuvants may be included in such formulations.

Alternatively, anti-*vpr* antibodies may be administered as therapeutics to treat individuals infected with HIV. The anti-*vpr* antibodies are preferably produced against eukaryotically-produced *vpr*. They are administered in an effective dose; i.e. a dose sufficient to inactivate some or all of the *vpr* present in the individual such that the progress of HIV in the individual is inhibited or otherwise reduced. Multiple doses may be administered. One having

Using PCR and recombinant DNA technology, truncation mutants of the *vpr* gene were constructed and cloned into pBABE expression plasmids. These constructs delete *vpr* in approximately 20AA groups from the carboxy terminus traveling in toward the amino terminus of the protein. The resulting protein products are 72AA, 50AA and 30AA.

Preliminary studies indicate that the carboxyl terminus 24AA of *vpr* is necessary for induction of differentiation of both the rhabdomyosarcoma and glial cell lineages as loss of the inhibition of proliferation and loss of morphological changes with the deletion mutants has been observed. One interesting observation of these studies is that this carboxy region contains a significant region of homology with the muscle oncogene *ski*. The avian retroviral oncogene *ski* shows properties resembling those described for *vpr* (Colmenares and Stavnezer, Cell, 1989).

Studies suggest that carboxy terminal deletion *vpr* mutants still retain *gag* binding activity in this system. This assay therefore differentiates the functional region of *vpr* which interacts with *gag* and the functional region for cell differentiation function.

**Example 12: Antibodies and Immunizations**

Rabbit anti-*vpr* peptide serum (Garrett, *et al.*, *J. Virol.*, **1991**, *65*, 1653) (a.a. 2-21: Cys-Glu-Gln-Ala-Pro-Glu-Asp-Gln-Gly-Pro-Gln-Arg-Glu-Pro-His-Asn-Glu-Trp-Thr-Leu-Glu; SEQ ID NO:4) was obtained from Dr. Brian Cullen through the NIH AIDS Research and Reference Reagent Program. To produce additional rabbit antibodies against *vpr*, a rabbit was immunized with 10-20 μg of partially purified *vpr* protein (produced as described below from the anti-*vpr* column) in complete Freund's adjuvant (CFA) once, then with incomplete adjuvant (IFA) for subsequent immunizations. Final immunization was with 50 μg of each of three keyhole limpet hemocyanin (KLH)-coupled *vpr* peptides in IFA. Peptides were purchased from American Bio-Technologies. Sequences of peptides: *vpr* 9-20 (Gly-Pro-Gln-Arg-Glu-Pro-His-Asn-Glu-Trp-

Thr-Leu; SEQ ID NO:5), 41-55 (Gly-Leu-Gly-Gln-His-Ile-Tyr-Glu-Thr-Gly-Asp-Thr-Trp-Ala; SEQ ID NO:6), 81-96 (Ile-Gly-Val-Thr-Gln-Gln-Arg-Arg-Gln-Arg-Asp-Gly-Ala-Ser-Arg-Ser; SEQ ID NO:7). To produce mouse anti-*vpr* serum, Balb-c mice were immunized with 20 μg of single peptides coupled to KLH in CFA for the first immunization and IFA for subsequent immunizations.

**Example 13: Column Chromatography**

Affinity columns were constructed according to Harlow and Lane. Harlow, E. and Lane, E., *Antibodies: A Laboratory Manual*, 1988, Cold Spring Harbor Laboratory Press which is incorporated herein by reference. The IgG fraction of 250 μl of the rabbit peptide serum was bound to 1 ml of protein A agarose beads (Gibco BRL), washed in 0.2 M sodium borate buffer (pH 9.0) and coupled with 20 mM dimethylpimelimidate (DMP). The polyclonal rabbit anti-*vpr* column was constructed according to the same procedure using 6 ml of serum and 3 ml of protein G agarose beads (Gibco BRL).

**Example 14: Detection of Anti-*vpr* Antibodies by Capture ELISA**

For detection of anti-*vpr* antibodies, an ELISA was performed using eukaryotically-produced *vpr* attached to solid phase, followed by the addition of the test sample. Peroxidase-coupled anti-human antibody was used for detection (Boehringer Mannheim). Color development was with 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) (Sigma) according to the manufacturer's instructions. Anti-p24 antibodies were detected using recombinant p24 (American BioTechnologies) attached to solid phase support. Both recombinant proteins were used at an approximate concentration of 1 μg/ml, 50 μul/well. Incubation was done for 1 hour at 37° or 12 hours at 4°. Detection antibodies were used at a 1:15000 dilution as per manufacturers directions.

**Example 15: Detection of *vpr* by Capture ELISA**

For detection of *vpr*, a capture ELISA was performed. Rabbit anti-*vpr* peptide serum (reactive to aa 2-21) was immobilized in wells of a 96-well ELISA plate (Immulon II, Dynatech) in carbonate-bicarbonate buffer (0.2 M, pH 9.2). Detection of bound antigen was performed using a mouse anti-

cells exposed continuously to 20% *vpr* supernatant ceased proliferation after approximately 3 days and after 7-9 days underwent morphological differentiation similar to that observed following transfection with the HIV-1 *vpr* gene, including great enlargement, the presence of long processes similar to myotubes, multinucleation and cessation of proliferation. Exposure to greater than 20% supernatant proved to be rapidly fatal to the cells. Incubation of TE671 cells with equal concentrations of control supernatants failed to induce any of these changes. Cells exposed to *vpr* for 3 days were examined for the presence of adult muscle myosin, a differentiation marker for these cells (Aguanno *et al.*, *Cancer Res.*, **1990**, *50*, 3377). Greater than 90% of cells stained positive using an anti-myosin antibody, demonstrating that the differentiation was along the pathway to which to these cells are committed and is identical to that induced by expression of *vpr* from within.

**Example 31**

Melanoma cells were transfected with a nucleic acid molecules that comprised a nucleotide sequence that encoded *vpr*. In test experiments, transfected cells were selected and implanted into mice. As a control, untransfected melanoma cells were introduced into other mice.

The mice with implanted transfected melanoma cells displayed a vast reduction in the number of tumors developing from the injected cells as compared to the number of tumors developing in control mice. The results from these experiments clearly demonstrated that while *vpr* did not complete eliminate the tumorigenicity of the melanoma cells, the presence of the *vpr* gene in the melanoma cells significantly and substantially reduced the tumorigenicity of the cells.

**Example 32**

Three peptides were synthesized by routine methods. each peptide is an immunogenic fragment of *vpr* protein. The first, SEQ ID NO:8, is residues 9-20. The second, SEQ ID

NO:9, is residues 41-55. The third, SEQ ID NO:10, is residues 81-96.

Each peptide has been confirmed to be immunogenic in rabbits and mice. The antibodies produced cross-react to *vpr*.

Pharmaceutical compositions are produced which comprise SEQ ID NO:8, SEQ ID NO:9 and/or SEQ ID NO:10 and a pharmaceutically acceptable carrier or diluent. Administration of such pharmaceutical compositions in an individual suffering from HIV infection results in a immunogenic response that produces antibodies which cross react with *vpr*. The antibodies thus produced bind to and inactivate *vpr* present as a result of the HIV infection.

US005827723A

# United States Patent [19]

Matsushita

[11] Patent Number: 5,827,723

[45] Date of Patent: Oct. 27, 1998

[54] **NEUTRALIZING MONOCLONAL ANTIBODY 0.5β WHICH BINDS AN EPITOPE LOCATED WITHIN THE REGION OF AMINO ACIDS 308-331 OF HTLVIIB GP120**

[75] Inventor: **Shuzo Matsushita**, Kumamoto-ken, Japan

[73] Assignee: **Kyowa Hakko Kogyo Co. Ltd.**, Tokyo, Japan

[21] Appl. No.: **972,890**

[22] Filed: **Nov. 6, 1992**

**Related U.S. Application Data**

[63] Continuation of Ser. No. 618,033, Nov. 27, 1990, abandoned, which is a continuation of Ser. No. 198,957, May 26, 1988, abandoned.

[30] **Foreign Application Priority Data**

Jul. 29, 1987 [JP] Japan ..... 62-133909

[51] **Int. Cl.**[6] ..... **C12N 5/20**; C07K 16/08; G01N 33/53

[52] **U.S. Cl.** ..... **435/240.27**; 530/388.35; 435/78.21

[58] **Field of Search** ..... 530/388.35; 435/172.2, 435/70.21, 240.27, 7.21; 424/85.8, 148.1

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*Primary Examiner*—Paula K. Hutzell
*Attorney, Agent, or Firm*—Schweitzer Cornman & Gross

[57] **ABSTRACT**

The present invention relates to monoclonal antibody 0.5β which binds to an epitope located within the region of amino acids 308–331 of HTLVIIIB gp120 and is capable of substantially neutralising the activity of human immunodeficiency viruses, to a hybridoma which produces the 0.5β antibody, to processes for preparing them and to compositions containing an effective amount of the antibody.

**3 Claims, 4 Drawing Sheets**

COL 5 PHARMACEUTICALS / MABS

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0022-538X/91/031653-05$02.00/0

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# Rev Activates Expression of the Human Immunodeficiency Virus Type 1 *vif* and *vpr* Gene Products

ELIZABETH D. GARRETT, LAURENCE S. TILEY, AND BRYAN R. CULLEN*

*Howard Hughes Medical Institute and Department of Microbiology and Immunology, Duke University Medical Center, Durham, North Carolina 27710*

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**The proteins encoded by human immunodeficiency virus type 1 (HIV-1) can be divided into two temporally regulated classes. Early gene products are encoded by multiply spliced mRNA species and are expressed constitutively. In contrast, late proteins are encoded by a class of unspliced or singly spliced viral transcripts whose cytoplasmic expression is induced by the viral Rev *trans* activator. Here, we demonstrate that the viral Vif and Vpr proteins are encoded by singly spliced viral mRNAs whose expression is activated by Rev. This activation is shown to result from the reduced utilization of splice sites adjacent to or within the *vif* and *vpr* coding sequences. Vif and Vpr therefore belong to the class of late HIV-1 gene products.**

The pathogenic retrovirus human immunodeficiency virus type 1 (HIV-1) displays a high level of genetic complexity (Fig. 1). In addition to carrying the *gag*, *pol*, and *env* genes characteristic of all known replication-competent retroviruses, HIV-1 encodes at least six auxiliary proteins. Two of these, termed Tat and Rev, are essential *trans* regulators of viral gene expression, while two others, termed Vpu and Vif, have been shown to be important for the morphogenesis and release of infectious virions (reviewed in reference 5). The roles of the viral *nef* and *vpr* gene products remain less clear, as both are dispensable for efficient HIV-1 replication in culture (3, 10, 16). However, recent data suggest that Vpr may be a virion structural protein whose expression modestly enhances the rate of viral replication (2, 3, 16).

The nine HIV-1 proteins enumerated or alluded to above are encoded by more than 20 distinct mRNA species that are derived from the posttranscriptional processing of the initial, genome-length viral transcript (7, 14, 19–21). The pattern of expression of these HIV-1 mRNAs displays a marked temporal regulation (9). In the early stages of infection, viral gene expression is limited to the small, multiply spliced mRNA species that are known to express the viral regulatory proteins Tat and Rev as well as the *nef* gene product (Fig. 1) (6, 7, 9). It is hypothesized (9, 17) that accumulation of the Rev protein to a critical level activates the expression of the unspliced and singly spliced mRNA species that encode the viral structural proteins Gag, Pol, and Env. HIV-1 proviruses lacking a functional *rev* gene product or bearing a defective copy of the *cis*-acting RNA target sequence for Rev, the Rev response element (RRE), are unable to progress to the late, structural phase of the viral replication cycle and remain locked in the early, regulatory phase (6, 7, 12).

Recently, it was demonstrated that Vpu is also a late gene product that is encoded by the same singly spliced bicistronic mRNA species as Env (21). However, the temporal regulation of the HIV-1 *vif* and *vpr* gene products has remained unclear. Schwartz et al. (20) have proposed that Vif and Vpr are encoded by two doubly spliced viral mRNA species containing the hypothetical coding exons designated E2A and E3A, respectively (Fig. 1D). These multiply spliced mRNAs, which would be structurally comparable to the known multiply spliced *tat* mRNAs (Fig. 1C), would lack an RRE and would therefore have to be expressed independently of Rev. Alternatively, Vif and Vpr could be encoded either predominantly or exclusively by mRNAs structurally comparable to the singly spliced viral mRNAs that encode Env and the truncated form of Tat (Fig. 1D) (18). In this case, the Vif and Vpr proteins would fall into the class of late viral gene products whose expression is activated by the Rev protein.

To examine the Rev dependence of HIV-1 *vif* gene expression, we transfected (4) cultures of the HIV-1 replication-permissive monkey cell line COS (11) with an expression plasmid containing a full-length, replication-competent HIV-1 provirus (pHIV-1) or with a similar HIV-1 provirus bearing a defective viral *rev* gene (pHIV-1ΔRev) (13). These expression plasmids are based on the HXB-3 proviral isolate and are similar to those previously described (13), except that the single-base-pair frame-shift mutation present within the *vpr* gene of the HXB-3 isolate (3, 15, 16) has been corrected in order to permit expression of the full-length *vpr* gene product. At 72 h after transfection, total cytoplasmic mRNA was harvested and subjected to Northern (RNA) analysis (12) with probes specific for the *tat* gene or the *vif* gene (Fig. 2). The HIV-1 *tat* gene is known to be expressed as a set of multiply spliced, ~2-kb transcripts in the absence of Rev and as a singly spliced, ~4-kb transcript in the presence of Rev (Fig. 1C) (7, 14, 20). This result is confirmed in Fig. 2, which shows a marked accumulation of exclusively multiply spliced *tat* mRNAs in the absence of Rev (Fig. 2, lane 2). In the presence of Rev, expression of the 2-kb *tat* mRNAs is reduced whereas the singly spliced *tat* mRNA, as well as the genomic transcript, is readily detected. In contrast, the probe specific for the *vif* open reading frame failed to detect any specific viral mRNA species in cells transfected with the pHIV-1ΔRev construct (Fig. 2, lane 5). Even with prolonged exposure, no specific mRNA species could be detected in this lane (data not shown). However, both a singly spliced and a genome-length viral RNA species were detected by the *vif*-specific probe in cells transfected with the wild-type HIV-1 proviral expression plasmid (Fig. 2, lane 6). Of note, the singly spliced mRNA detected by the *vif*-specific probe was observed to migrate more slowly than the predominant singly spliced species detected by the *tat*

* Corresponding author.



X-UPR (2-21)

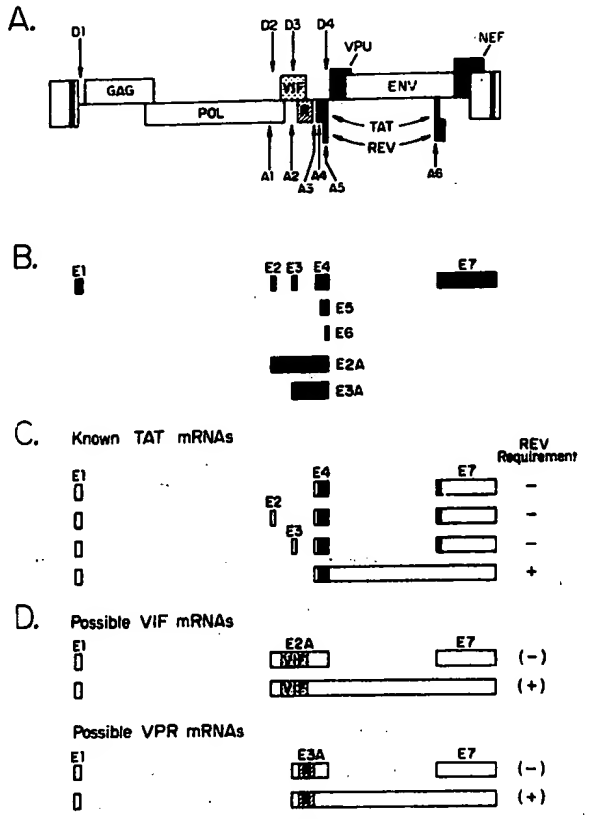


FIG. 1. RNA expression strategies in HIV-1. (A) Genetic organization of the HIV-1 provirus, showing the location of major splice donors (D1 to D4) and splice acceptors (A1 to A6). R, *vpr*. (B) HIV-1 exons observed in the multiply spliced viral mRNA species. E1 to E7 represent exons that have been cloned and characterized. E2A and E3A represent hypothetical exons proposed by Schwartz et al. (20). The exon nomenclature used here is similar to that previously proposed (14, 20). (C) HIV-1 Tat protein occurs in two forms (7, 12). A full-length, two-exon form of Tat is encoded by a set of at least three different multiply spliced mRNAs and is expressed independently of Rev (12, 19, 20). In the presence of Rev, a singly spliced Tat mRNA that encodes a truncated, one-exon form of Tat (7, 12) is observed. (D) By analogy with the Tat protein, the HIV-1 *vif* and *vpr* gene products could be expressed from doubly spliced mRNA species containing the hypothetical E2A and E3A exons (20). Expression of these mRNAs, which would lack the *env* gene-specific RRE signal, would therefore be predicted to be Rev independent. In contrast, Vif and Vpr might be translated entirely from singly spliced mRNAs (18) whose cytoplasmic expression would be dependent on Rev.

probe, which was predicted by their projected sizes of ~5.0 and ~4.1 kb, respectively (Fig. 1). Results obtained with a probe specific for the HIV-1 *vpr* gene were similar to those obtained with the *vif*-specific probe (data not shown).

The Northern analysis whose results are presented in Fig. 2 suggests that *vif* is detectably expressed only as a singly spliced, ~5-kb transcript and only in the presence of Rev. One possible explanation for this result is that Rev induces the utilization of the *vif* gene-specific A1 splice acceptor (Fig. 1). Alternatively, Rev might activate Vif expression by reducing utilization of the D2 splice donor in a population of RNA that has already completed the D1-A1 splice. It is of interest that such a splicing event would generate the small

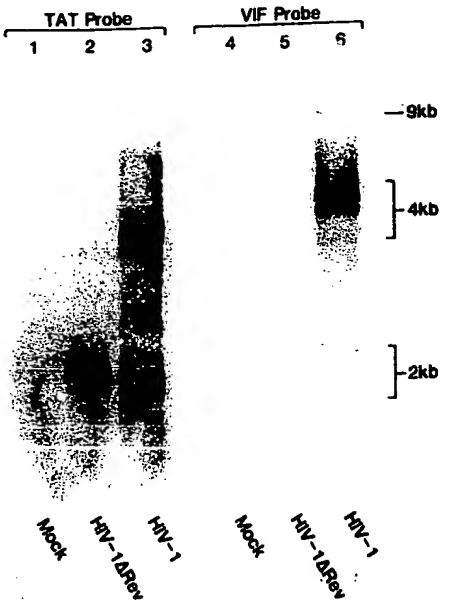


FIG. 2. Northern analysis of HIV-1 *tat* and *vif* mRNAs. COS cell cultures were transfected (4) with an intact HIV-1 provirus, with a provirus bearing a defective Rev gene (pHIV-1ΔRev), or with a negative control vector (mock transfected). Total cytoplasmic RNA was isolated (12) 72 h after transfection, and Northern analysis was performed as described previously (12), with 10 μg of RNA per lane. The nitrocellulose filter was initially analyzed with a probe specific for the first (E4) exon of *tat* (proviral coordinates 5794 to 5955; see reference 15) (lanes 1 to 3). Subsequently, the filter was stripped and rehybridized with a probe specific for the viral *vif* gene (proviral coordinates 5077 to 5332) (lanes 4 to 6). The approximate sizes of the unspliced (~9 kb), singly spliced (~4 kb), and multiply spliced (~2 kb) viral mRNAs, as determined by their mobilities, are indicated.

noncoding exon, designated E2 in Fig. 1, that has previously been detected in the multiply spliced HIV-1 mRNA species (14, 19, 20). To distinguish between these possible explanations, we subjected the cytoplasmic mRNA samples described above to quantitative S1 nuclease analysis (Fig. 3).

Figure 3a illustrates a probe, end labeled in the first exon of the *tat* gene, that spans the D4 splice site. This probe can therefore detect all cytoplasmic viral mRNAs containing exon E4 and will quantitate the utilization of the D4 splice donor in this population. In cells transfected with pHIV-1ΔRev (Fig. 3A, lane 2), essentially all of the cytoplasmic HIV-1 transcripts detected by this probe were spliced at the D4 site. In contrast, a large majority of the viral RNAs detected in the culture transfected with a wild-type HIV-1 provirus were unspliced at the D4 site (Fig. 3A, lane 3). This result therefore confirms those of a previous study (12) showing that coexpression of Rev reduces the utilization of the D4 splice site.

Figure 3b illustrates a second end-labeled probe that is designed to detect only those mRNAs that have already made the splice from the D1 splice donor to the A1 splice acceptor, i.e., mRNAs which could present the *vif* gene as the first open reading frame. This probe also spans the D2 splice site. It is therefore possible to quantitate the level of utilization of the D2 splice donor in a population of mRNAs that has already made the D1-A1 splice. Only mRNAs not spliced at D2 could encode the HIV-1 Vif protein. In fact, very little RNA not spliced at the D2 splice site (i.e., *vif*

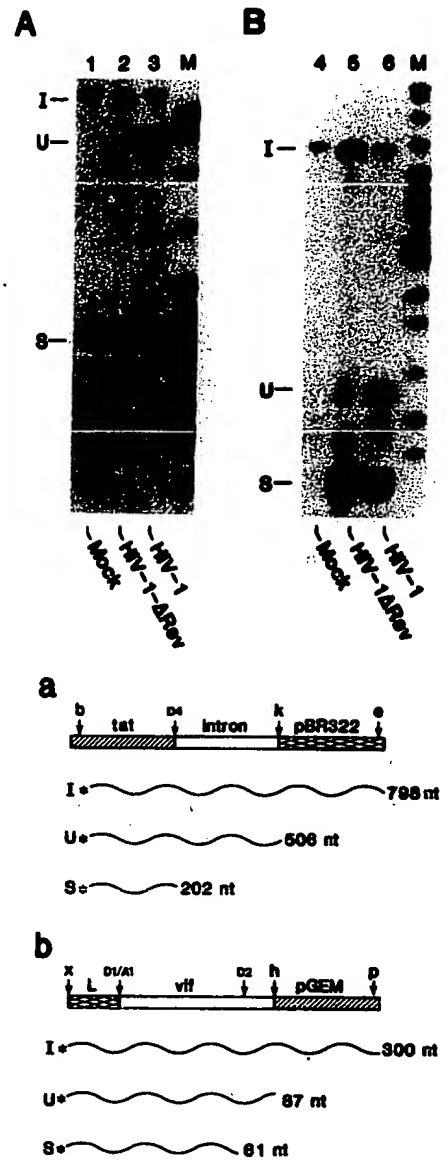


FIG. 3. S1 nuclease analysis of HIV-1 *tat* and *vif* mRNAs. The RNA samples used in this experiment were identical to those used for Northern analysis (Fig. 2). Quantitative S1 nuclease analyses were performed as described elsewhere (4, 12), with 5 μg of total cytoplasmic RNA per lane. (A, a) The probe strategy used in this experiment has been described previously (12). The probe used was end labeled at a *Bst*Y1 (b) site within the first coding exon of *tat* and extends through splice donor D4 into the *env* gene-specific intron. A pBR322 DNA tag was attached at an intron *Kpn*I (k) site to allow us to distinguish the full-length input (I) probe from probe fragments rescued by viral transcripts that were unspliced (U) or spliced (S) at the D4 splice donor. Size markers (M) were obtained by end labeling an *Msp*I digest of pBR322 DNA. e, *Eco*RI. (B, b) To quantitate the level of splicing at the D2 splice acceptor in HIV-1 transcripts already spliced at the A1 and D1 splice sites, we used the polymerase chain reaction to generate a molecular clone that contained the D1-A1 splice junction. The 5′ primer used was homologous to HIV-1 sequences located 3′ to the A1 splice acceptor (positions 4913 to 4927), and it also contained a 12-bp 5′ extension homologous to HIV-1 leader sequences located 5′ to the D1 splice donor (positions 732 to 743). The 3′ primer spanned a *Hin*dIII (h) site located 26 nucleotides (nt) 3′ to the D2 splice acceptor. This polymerase chain reaction fragment was cloned into the pGem3zf(+) polylinker (Promega). The probe used was end labeled at an *Xma*I (X) site introduced into the HIV-1 leader (L) sequence and extends through the D2 splice donor into the *vif* open reading frame. A pGem DNA tag was attached at a *vif* gene *Hin*dIII site to allow us to distinguish the full-length input (I) probe from probe fragments rescued by RNA species that were unspliced (U) or spliced (S) at the D2 splice donor. p, *Pvu*II.

mRNA) was detected in cells transfected with pHIV-1ΔRev (Fig. 3B, lane 5). Instead, we detected a high level of RNA spliced at the known D2 splice site as well as a lower level of a slightly larger RNA spliced at an immediately adjacent splice donor that we term D2*. Utilization of this second 5′ splice site was detected in several independent experiments, although always at a lower level than that of the known D2 site, and it therefore appears probable that this represents a novel minor splice donor present in the HIV-1 provirus. S1 nuclease analysis maps the location of this site to 14 nucleotides 3′ to the known D2 site. The D2* sequence is therefore 5′-A/GUAAU-3′, which is similar to the splice donor consensus sequence (5′-G/GUAAG-3′) (1). However, the significance of this novel 5′ splice site, which should result in a slightly larger noncoding E2 exon, is unclear.

In cells transfected with a wild-type HIV-1 provirus, a marked increase in the level of mRNA unspliced at the D2 site (i.e., *vif* mRNA) was detected, as was a concomitant drop in the level of mRNA spliced at either D2 or D2* (Fig. 3B, lane 6). This result therefore supports the hypothesis that Rev activates the expression of *vif* mRNA by reducing the utilization of the D2 splice donor.

The experiments presented thus far have examined Vif and Vpr expression entirely at the RNA level. To more directly test whether the expression of the Vif and Vpr proteins depends on Rev coexpression, we raised rabbit antisera specific for these viral proteins by using as an immunogen either a peptide (N-CEQAPEDQGPQREPHNE WTLE-C) identical to amino acids 2 to 21 of the predicted *vpr* open reading frame or a full-length recombinant Vif protein obtained from the National Institutes of Health AIDS Reagent Program. We then used these antisera, together with a previously described (12) rabbit antiserum specific for the HIV-1 Tat protein, to analyze the expression of Vpr, Vif, and Tat proteins in transfected cells. For this purpose, cultures transfected with pHIV-1, pHIV-1ΔRev, or a control plasmid were metabolically labeled with [$^{35}$S]cysteine and [$^{35}$S]methionine (12). The labeled proteins were then extracted (12), and equal aliquots were subjected to immunoprecipitation analysis with anti-Tat, anti-Vpr, or anti-Vif rabbit antisera. Precipitated proteins were resolved by electrophoresis through a denaturing sodium dodecyl sulfate (SDS)–14% polyacrylamide gel and were then visualized by autoradiography (Fig. 4).

As predicted, the culture transfected with pHIV-1ΔRev gave rise to a high level of the two-exon form of Tat protein (Fig. 4, lane 5) encoded by the various multiply spliced *tat* mRNA species (Fig. 1B) (20). Coexpression of Rev resulted in a marked reduction in the expression of the full-length Tat protein and an induction of the one-exon form of Tat (Fig. 4, lane 6) encoded by the singly spliced *tat* mRNA (Fig. 1C) (12, 20). The culture transfected with the wild-type HIV-1 proviral clone also gave rise to a specific band (~14 kDa) with the mobility predicted previously (2) for the HIV-1 Vpr protein (Fig. 4, lane 3). However, no Vpr protein was detected in the absence of Rev coexpression (Fig. 4, lane 2).

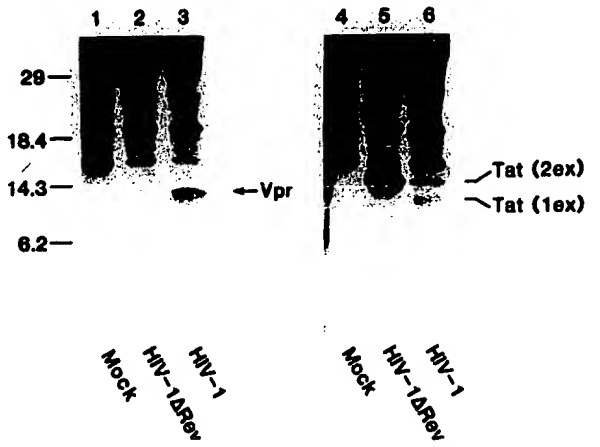


FIG. 4. Immunoprecipitation analysis of COS cell cultures transfected with proviral clones. COS cell cultures were transfected with the wild-type HIV-1 provirus or with a provirus bearing a defective *rev* gene (pHIV-1ΔRev) or were mock transfected with a control plasmid, pBC12/CMV, as indicated. At 72 h posttransfection, cultures were radiolabeled and lysed (12), and 800-μl aliquots of the solubilized proteins were subjected to immunoprecipitation (12) with 8 μl of anti-Vpr antiserum (lanes 1 to 3) or 5 μl of anti-Tat antiserum (lanes 4 to 6). The precipitated proteins were resolved by electrophoresis. The relative mobilities of $^{14}$C-labeled protein molecular size standards (Bethesda Research Labs, Inc.) are indicated by their molecular masses in kilodaltons on the left. Vpr has been shown to migrate at ~14 kDa (2), while the two-exon (2ex) and one-exon (1ex) forms of Tat migrate at ~15.5 and ~14 kDa, respectively (12).

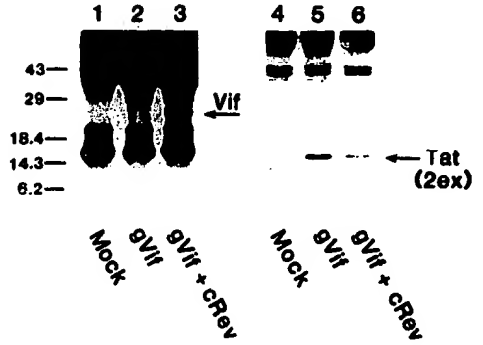


FIG. 5. Immunoprecipitation of the HIV-1 Vif protein from transfected cells. Immunoprecipitations were performed as described for Fig. 4. The *vif* gene expression plasmid pgVif contains HIV-1 proviral sequences extending from immediately 3′ to the A1 splice acceptor (Fig. 1A) to immediately 3′ to the end of the second coding exon of *tat* (positions 4917 to 8475). This plasmid therefore contains a truncated, defective *rev* gene but includes an intact RRE. These HIV-1 sequences were placed under the control of the cytomegalovirus immediate-early promoter in the pBC12/CMV plasmid (4). Cultures were transfected with pgVif together with a Rev expression plasmid (pcRev) or a control plasmid (pBC12/CMV). Negative control (mock) cultures were transfected with pBC12/CMV alone. Labeled cell extracts (800-μl samples) were subjected to immunoprecipitation with 8 μl of anti-Vif antiserum (lanes 1 to 3) or 5 μl of anti-Tat antiserum (lanes 4 to 6). The Vif protein has been shown to migrate at ~23 kDa on SDS-polyacrylamide gels (8).

Therefore, Vpr expression, like expression of the one-exon form of Tat, is dependent on Rev.

Unfortunately, it proved impossible to detect Vif protein in cultures transfected with HIV-1 proviral clones by using the rabbit anti-Vif antibody at our disposal (data not shown). In an attempt to increase expression to a detectable level, we constructed an expression plasmid, pgVif, in which a segment of the HIV-1 genome extending from immediately 3′ to the A1 splice acceptor to immediately 3′ to the end of the second exon of Tat was inserted into the expression plasmid pBC12/CMV (4). This segment of the HIV-1 provirus contains an intact viral RRE but lacks a functional *rev* gene. Unspliced mRNAs transcribed from this plasmid are predicted to be structurally comparable to the singly spliced *vif* mRNA and should, therefore, direct the synthesis of Vif protein. In contrast, fully spliced mRNAs derived from pgVif are predicted to encode predominantly the two-exon form of the HIV-1 Tat protein. In fact, a culture transfected with pgVif alone produced no detectable Vif protein (Fig. 5, lane 2) but yielded a high level of two-exon Tat (Fig. 5, lane 5). In contrast, coexpression of Rev in *trans* resulted in the induction of the 23-kDa Vif protein (Fig. 5, lane 3) (8, 22) and reduced synthesis of the 2-exon form of viral Tat protein (Fig. 5, lane 6).

In this report, we have demonstrated that expression of the HIV-1 Vif and Vpr proteins is activated by and dependent upon coexpression of the HIV-1 Rev protein. These results were obtained by the transfection of full-length HIV-1 proviral clones into the monkey cell line COS. Although COS cells are permissive for HIV-1 replication (11), they do not represent a physiologically relevant host cell for the HIV-1 virus. Nevertheless, these results clearly predict that Vif and Vpr expression is likely to be activated late in the HIV-1 replication cycle in infected primary human T cells or macrophages.

We thank Sharon Goodwin for secretarial assistance and Mildred McAdams and Richard Randall for synthesis of the Vpr peptide. Recombinant Vif protein was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

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# Speed of Progression to AIDS and Degree of Antibody Response to Accessory Gene Products of HIV-1

**Peter Reiss, Joep M.A. Lange, Anthony de Ronde, Frank de Wolf, John Dekker, Christine Debouck, and Jaap Goudsmit**

*Departments of Virology (J.M.A.L., A.de R., F.de W., J.D., J.G.) and Internal Medicine (P.R., J.M.A.L.) (Academic Medical Centre), University of Amsterdam, the Netherlands; and the Department of Molecular Genetics, Smith Kline and French Laboratories, King of Prussia, Pennsylvania (C.D.)*

Antibodies to *E. coli*-produced HIV-1 *nef, rev, tat, vpu,* and *vpr* proteins were measured by enzyme immunoassay in serial sets of sera from 72 men seroconverting for antibodies to HIV-1 structural proteins, and from 190 initially symptom-free men who were seropositive for these antibodies at entry into the study. In the men seroconverting for antibodies to structural proteins the levels of *nef-, rev-,* and *tat*-specific antibodies, but not of *vpu-,* and *vpr*-specific antibodies, within 3 months of seroconversion, appeared to be lower in the five men progressing to AIDS, compared with the men remaining symptom-free during follow-up. Analysis of the prevalence of previously described antibody profiles to these accessory gene products was carried out. In all HIV-1 antibody seroconverters and in those HIV-1 antibody seropositive men with 15 or more months of follow-up who progressed to AIDS, there was a shift from predominantly *nef-* and *vpu*-specific antibody negative profiles in the men developing AIDS in the early years of the study to predominantly *nef-* and *vpu*-specific antibody positive profiles in men who developed AIDS later. *Rev-* and *tat*-specific antibody negative profiles were dominant in men progressing to AIDS throughout follow-up. No *vpr*-specific antibody profile occurred preferentially in the men progressing to AIDS throughout follow-up. Low antibody reactivity to accessory gene products *nef, rev,* and *tat* appears, like low anti-core antibody reactivity, to be associated with progression to AIDS relatively rapidly after infection with HIV-1.

**KEY WORDS:** HIV-1, rapid disease progression, serological markers

## INTRODUCTION

The genome of HIV-1 contains genes coding for viral structural proteins as well as accessory genes [Gallo et al., 1988]. The latter genes designated *nef, rev, tat, vif, vpu,* and *vpr,* code for proteins some of which have been shown to regulate viral replication in vitro. Antibodies to each of the accessory gene products have been described in HIV-1-infected individuals [Arya and Gallo, 1986; Franchini et al., 1987; Barone et al., 1986; Krone et al., 1988; Reiss et al., 1989a,b,c; Chanda et al., 1988; Allan et al., 1985; Ameisen et al., 1989a,b; Kan et al., 1986; Lee et al., 1986; Wong-Staal et al., 1987; Matsuda et al., 1988; Cohen et al., 1988; Strebel et al., 1988; Sabatier et al., 1989; Cheingsong-Popov et al., 1989], but the seroprevalence varies from high in the case of antibodies to *nef* [Ronde de et al., 1988; Sabatier et al., 1989; Reiss et al., 1989a; Cheingsong-Popov et al., 1989] and to low in the case of anti-*tat* [Barone et al., 1986; Krone et al., 1988; Reiss et al., 1989b], to intermediate for anti-*rev* [Reiss et al., 1989b,c], anti-*vpu* [Matsuda et al., 1988; Reiss et al., in press], and anti-*vpr* [Wong-Staal et al., 1987; Reiss et al., in press]. We have previously described five patterns of antibody response to recombinant *E. coli*-produced *nef, rev, tat, vpu,* and *vpr,* in prospectively collected serum samples from a cohort of HIV-1-infected homosexual men, consisting both of men with documented seroconversion for antibodies to HIV-1 structural proteins *(gag/env)* and of men who were HIV-1 antibody seropositive at study entry [Reiss et al., 1989a,c in press]. Antibodies to the accessory gene products could be persistently detectable from the time of *gag/env* seroconversion, either at study entry or within 12 months. Alternatively, antibodies to the accessory gene products were only transiently or intermittently detectable, or not detectable at all. More cases of AIDS occurred in subjects who were persistently negative, or transiently or intermit-

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Address reprint requests to Prof. Dr. Jaap Goudsmit, Human Retrovirus Laboratory L1-157, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands.

tently positive for anti-*nef,* anti-*rev,* or anti-*tat,* compared with subjects who were or became persistently positive for these antibodies. No associations with clinical outcome were found for patterns of *vpu-* or *vpr*-specific antibodies. According to a recent report, high initial titres of anti-*nef* antibodies after HIV-1 antibody seroconversion correlated with lack of progression to AIDS [Cheingsong-Popov et al., 1989]. In view of these findings, we compare here the levels of antibody response to *nef, rev, tat, vpu,* and *vpr* between the subjects from our cohort with documented seroconversion for antibodies to *gag/env* who progressed to AIDS and those who remained symptom-free.

In a recent study in our cohort of HIV-1-infected men, the association of certain serological and immunological markers with progression to AIDS was found to be dependent on the duration of infection with HIV-1 [Wolf de et al., 1989]. The association of the absence or disappearance of antibodies to HIV-1 core proteins with disease progression was more pronounced in subjects progressing to AIDS early after infection, compared with subjects who progressed to AIDS later, after infection with HIV-1. In order to see whether a similar time dependency existed regarding associations of *nef-, rev-, tat-, vpu-,* and *vpr*-specific antibody profiles and disease progression, we compared the prevalence of the different profiles of these antibodies between the subjects who progressed to AIDS during different consecutive years after the start of the study.

## SUBJECTS AND METHODS

### Study Population

Between October, 1984 and March, 1986, 961 asymptomatic men, living in and around Amsterdam and with at least two homosexual contacts in the preceding 6 months, were enrolled in a prospective study on the prevalence and incidence of HIV infection and risk factors for AIDS. Epidemiological and clinical data were collected, and blood was sampled every 3 months [Wolf de et al., 1988].

In the first serum sample taken, 723 men were found to be seronegative for HIV-1 antibody, and 238 were found to be seropositive, using a commercially available enzyme immunoassay (EIA) based on purified human T-lymphotropic virus type III B as antigen (Vironostika, Organon Teknika, Oss, The Netherlands). Seropositivity was confirmed by immunoblotting as previously described [Lange et al., 1986]. During follow-up until April, 1988, 76 of the men initially seronegative for HIV-1 antibody seroconverted, as shown by a commercially available EIA based on *gag-* and *env*-encoded protein fragments (recombinant HIV EIA, Abbott Laboratories, North Chicago, IL). All available stored, sequentially taken, serum samples from subjects who were seropositive or who seroconverted for antibodies to HIV-1 structural proteins were tested retrospectively for antibodies to *nef, rev, tat, vpu,* and *vpr.* In four of the HIV-1 antibody seroconverters, insufficient sera were available for detection of accessory gene product-specific antibodies. One of these four men developed AIDS (CDC IV C1) 18 months after seroconversion. The mean duration of follow-up for the remaining 72 HIV-1 antibody seroconverters was 20.8 months (range 3–38 months). In 47 of the at-entry HIV-1 antibody seropositive men, follow-up was too short, and accessory gene product-specific antibody patterns could not be properly determined. In 7 of these 47 men, AIDS (CDC IV C1 in 3, CDC IV D in 4 men) was diagnosed 3–15 months after entry into the study. In another at-entry HIV-1 antibody seropositive man who developed AIDS (CDC IV C1) 18 months after study entry, accessory gene product-specific antibody patterns could not be determined because of lack of available serum samples. The mean duration of follow-up for the remaining 190 at-entry HIV-1 antibody seropositive men was 34.0 months (range 15–45 months).

Therefore for the present study sufficient data concerning accessory gene product-specific antibodies were available from 72 HIV-1 antibody seroconverters, 5 of whom had developed AIDS after a mean of 20.8 (range 11–30) months of follow-up, and from 190 at-entry seropositives, 28 of whom had developed AIDS after at least 15 months of follow-up (mean 26 months; range 15–39 months).

### Detection of Accessory Gene Product-Specific Antibodies

*Nef, rev, tat, vpu,* and *vpr* were produced in *E. coli* as galactokinase fusion proteins. The bacterially synthesized *nef, rev, tat, vpu,* and *vpr* were purified to greater than 95% homogeneity, and EIA was performed as described by Goudsmit et al. [1988], except that horseradish peroxidase-labeled goat anti-human IgG (KPL, Gaithersburg, MD) was used, instead of biotinylated goat anti-human IgG with streptavidin-biotinylated horseradish peroxidase complex. The last obtained serum samples of 100 of the longitudinally followed consistently HIV-1 antibody seronegative homosexual men were used as controls. A sample was considered to contain *nef-, rev-, tat-, vpu-,* and *vpr*-specific antibodies respectively if OD (sample) > OD (average of 100 HIV-1 antibody negative control samples) + 4 SD. Thus calculated, the rounded-off cut-off OD values for *nef-, rev-, tat-, vpu-,* and *vpr*-specific antibodies were 350, 300, 450, 350, and 400, respectively. OD ratios were calculated as the measured OD divided by the cut-off for the respective accessory gene product-specific antibody assay.

### Classification of Patterns of Antibody Response to Accessory Gene Products in Serial Serum Samples From Men Seroconverting or Seropositive for Antibodies to HIV-1 Structural Proteins

For each of the five accessory gene products five different patterns (groups) were distinguished.

1. Accessory gene product-specific antibodies were detected in all samples (persistently positive; group 1).
2. No accessory gene product-specific antibodies were found at the time of *gag/env* seroconversion or entry into the study, but they subsequently appeared and remained detectable throughout follow-up (seroconversion for accessory gene product-specific antibodies; group 2).
3. Accessory gene product-specific antibodies could not be detected in any of the samples (persistently negative; group 3).
4. Accessory gene product-specific antibodies were found from the time of *gag/env* seroconversion or at entry into the study, but subsequently disappeared and remained undetectable throughout follow-up (transiently positive; group 4).
5. One or more samples in which accessory gene product-specific antibodies were detected were preceded and followed by samples in which no such antibodies were detected (intermittently positive; group 5).

In the HIV-1 antibody seroconverters belonging to groups 2 and 5, the median time to first detection of accessory gene product-specific antibodies was 1–6 months, depending on the type of antibody and usually was less than 12 months. There were four exceptions: one anti-*nef* seroconversion at 21 months, one anti-*rev* seroconversion at 32 months, and one anti-*tat* seroconversion at 18 months after *gag/env* seroconversion. In one subject who was intermittently positive for anti-*rev*, anti-*rev* was first detected 18 months after *gag/env* seroconversion. In the HIV-1 antibody seroconverters belonging to groups 4 and 5, the median duration of the periods during which accessory gene-specific antibodies were detectable was 1.5–16.5 months but usually was less than 12 months. There were five exceptions: in two men transient detection of anti-*vpr* for 15 and 18 months respectively; intermittent detection of anti-*nef*, anti-*rev*, and anti-*tat*, each in one different subject, for 17, 24, and 15 months, respectively. Therefore in order to classify the accessory gene product-specific antibody patterns reliably in the at-entry HIV-1 antibody seropositive men, only men with at least 15 months of follow-up were considered, thereby minimizing the possibility of misclassification.

As we have described previously [Reiss et al., 1989a,c, in press], the OD values for detectable accessory gene product-specific antibody in subjects with transiently or intermittently detectable antibodies were clearly lower than in subjects in whom accessory gene product-specific antibodies were or became persistently detectable. For this reason, when determining the prevalence of the various accessory gene product-specific antibody patterns in subjects who developed AIDS in different consecutive years after the start of the study, subjects with persistently detectable accessory gene product-specific antibodies (groups 1 and 2) were considered as a single group (*nef*-, *rev*-, *tat*-, *vpu*-, or *vpr*-specific antibody positive) as were subjects with transiently, or intermittently detectable, or non-detectable accessory gene product-specific antibodies (*nef*-, *rev*-, *tat*-, *vpu*-, or *vpr*-specific antibody negative).

### Clinical Classification

Subjects were assessed according to the Centers for Disease Control (CDC) criteria [Centers for Disease Control, 1986]. Patients with AIDS belonged to CDC IV C1 (22 patients), CDC IV D (10 patients, 9 with Kaposi's sarcoma and 1 with malignant lymphoma), or CDC IV B (1 patient with AIDS dementia).

### Statistics

Chi-square for trend was used when appropriate.

## RESULTS

### Levels of Antibody Response to *nef, rev, tat, vpu,* and *vpr* in the HIV-1 Antibody Seroconverting Men

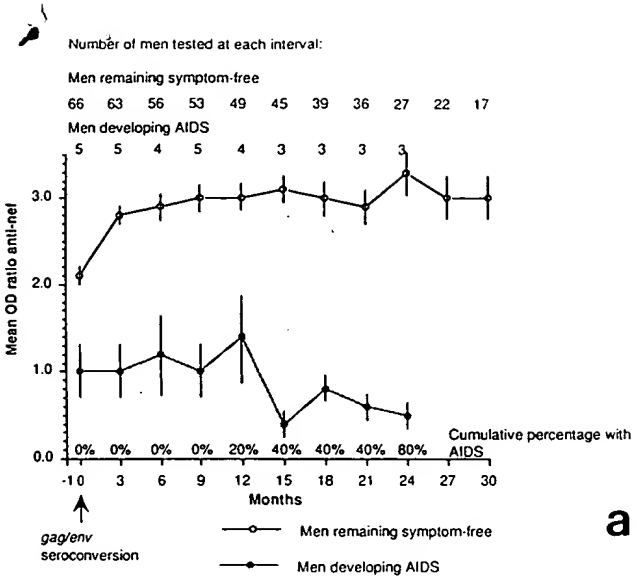
The mean OD ratios of antibodies to *nef, rev, tat, vpu,* and *vpr* in the 5 HIV-1 antibody seroconverters who developed AIDS compared with the 66 men who remained symptom-free during follow-up, are depicted in Fig. 1. One subject who developed CDC IV A 18 months after *gag/env* seroconversion was not included. In the case of antibodies to *nef, rev,* and *tat,* the mean OD ratios from the time of *gag/env* seroconversion or within 3 months thereafter were lower in the subjects developing AIDS than in subjects remaining symptom-free. All HIV-1 antibody seroconverters who developed AIDS during follow-up did so within 24 months after *gag/env* seroconversion, except for one in whom AIDS was diagnosed 30 months after *gag/env* seroconversion. Mean OD ratios of antibodies to *vpu* and *vpr* did not differ, early after infection, between subjects who developed AIDS and those who remained symptom-free.

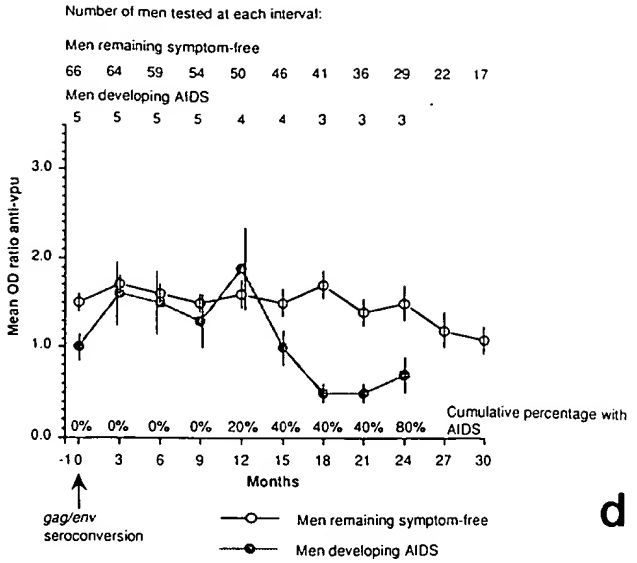
### Number of Cases of AIDS per Year, Related to the Profiles of Antibody Response to *nef, rev, tat, vpu,* and *vpr*

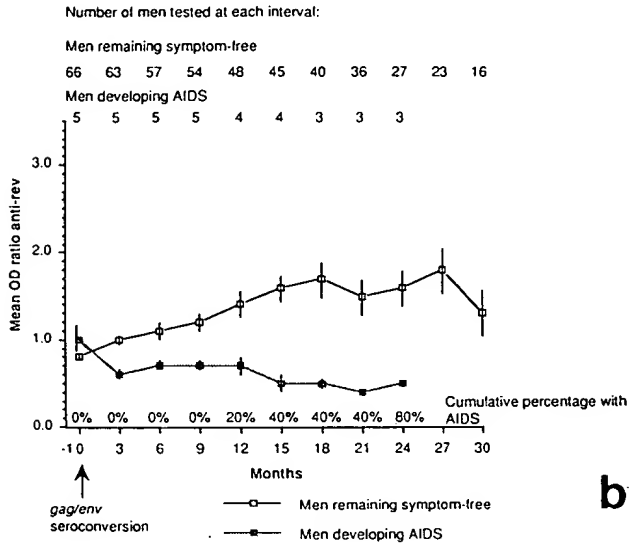
Although a rising trend was found in the percentage of subjects with *nef*-, *vpu*-, or *vpr*-specific antibody positive profiles who developed AIDS in 1988 compared with 1986 and 1987, this trend could not be demonstrated to be significant, possibly because numbers were too small. In the case of anti-*rev* and anti-*tat* cases of AIDS continued to be predominantly diagnosed in subjects with *rev*- or *tat*-specific antibody negative profiles, throughout all 3 years after the start of the study (Fig. 2).

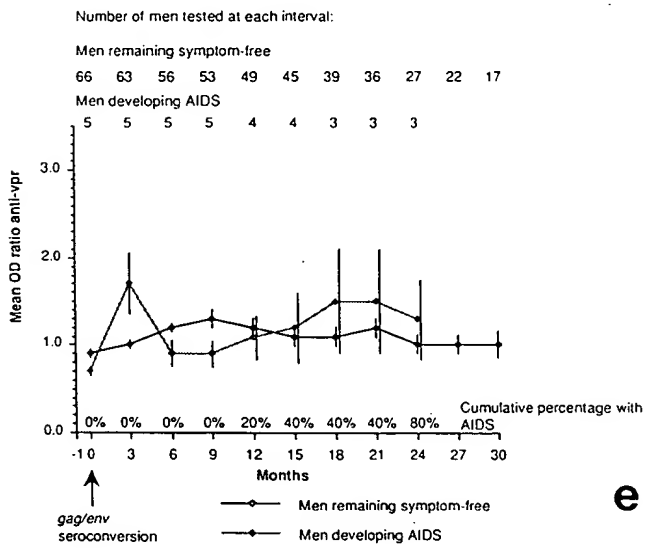
## DISCUSSION

Our data support the recent finding [Cheingsong-Popov et al., 1989] that high initial levels of antibodies to HIV-1 *nef* early after infection are associated with lack of rapid progression to AIDS. In addition, we found a similar association with respect to initial levels of antibodies to HIV-1 *rev* and *tat,* whereas no obvious differences in initial antibody response levels to HIV-1



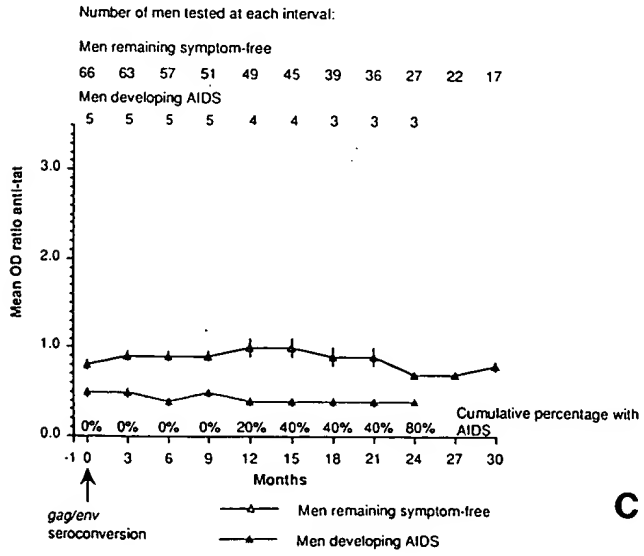




Fig. 1. Mean OD (optical density) ratios (mean of measured OD divided by cut-off OD of respective antibody assay) for *nef*- (A), rev- (B), *tat*- (C), *vpu*- (D), and *vpr*- (E)-specific antibodies, in HIV-1 antibody *(gag/env)* seroconverting men followed longitudinally, as well as cumulative incidence of AIDS in these men. All subjects were aligned at the moment of HIV-1 antibody seroconversion, which is represented by 0 months.

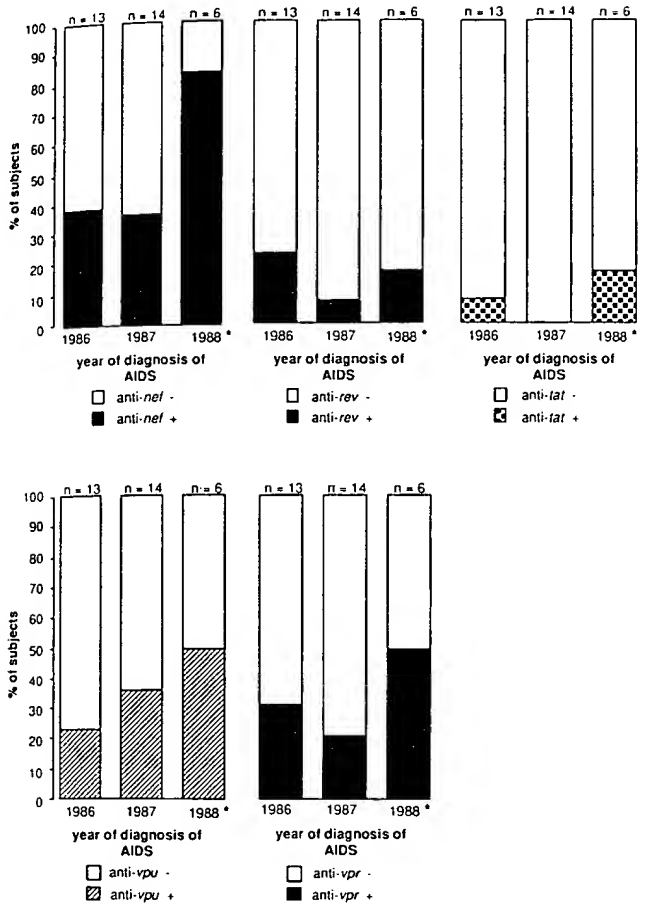



Fig. 2. Percentage of AIDS cases per year, related to profile of *nef*-, *rev*-, *tat*-, *vpu*-, and *vpr*-specific antibodies, respectively. *, first 3 months.

*vpu* and *vpr* were seen between subjects who did or did not progress to AIDS within the period of follow-up. Since the number of subjects in our cohort with documented HIV-1 antibody seroconversion who had progressed to AIDS was small, statistical validation of the above associations is not possible at this time, and therefore the conclusions remain speculative.

In previous reports, we found that a higher proportion of subjects progressed to AIDS, within the group of subjects with only transiently or intermittently or with no detectable *nef*- or *rev*-specific antibodies, in comparison with the group in which *nef*- or *rev*-specific antibodies were or became persistently detectable [Reiss et al., 1989a,c]. In the same cohort of HIV-1-infected men, similar results were obtained when analyzing *tat*-specific antibody responses (Reiss et al., unpublished observation).

Our present results indicate that, when analyzed by year of progression, as time goes by, cases of AIDS no longer occur preferably in *nef*-specific antibody negative, but rather in *nef*-specific antibody positive subjects. A similar though less marked trend is seen with respect to *vpu*-specific antibody profiles. These trends were not demonstrated to be statistically significant, which may well be due to the small numbers of cases of AIDS in 1988, during which year only the first 3 months were evaluated. With respect to *rev*- and *tat*-specific antibody profiles, the majority of cases of AIDS in the consecutive years after the start of the study continued to occur in subjects who were *rev*- or *tat*-specific antibody negative. Negative and positive *vpr*-specific antibody profiles were more equally distributed among AIDS cases without an obvious shift over time. In view of the above, prediction of the development of AIDS in subjects with a *nef*-specific antibody negative profile seems only to apply to subjects who progress to AIDS relatively rapidly after infection. This is in accordance with findings in the same cohort of men regarding the predictive value of absence or loss of anti-core antibody reactivity [Wolf de et al., 1989].

Since it is becoming increasingly evident that the vast majority of HIV-1-infected individuals will eventually develop HIV-1-related disease, markers identifying those who will progress relatively rapidly are important, in order for such subjects to benefit from early therapeutic intervention. We conclude that studying *nef*-, *rev*-, and *tat*-specific antibody levels and *nef*-specific antibody profiles may aid in identifying such individuals who are at high risk of early disease progression.

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# Extracellular Vpr Protein Increases Cellular Permissiveness to Human Immunodeficiency Virus Replication and Reactivates Virus from Latency

DAVID N. LEVY, YOSEF REFAELI,† AND DAVID B. WEINER*

*Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104-4283*

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**The *vpr* gene product of human immunodeficiency virus (HIV) and simian immunodeficiency virus is a virion-associated regulatory protein that has been shown using *vpr* mutant viruses to increase virus replication, particularly in monocytes/macrophages. We have previously shown that *vpr* can directly inhibit cell proliferation and induce cell differentiation, events linked to the control of HIV replication, and also that the replication of a *vpr* mutant but not that of wild-type HIV type 1 (HIV-1) was compatible with cellular proliferation (D. N. Levy, L. S. Fernandes, W. V. Williams, and D. B. Weiner, Cell 72:541–550, 1993). Here we show that purified recombinant Vpr protein, in concentrations of <100 pg/ml to 100 ng/ml, increases wild-type HIV-1 replication in newly infected transformed cell lines via a long-lasting increase in cellular permissiveness to HIV replication. The activity of extracellular Vpr protein could be completely inhibited by anti-Vpr antibodies. Extracellular Vpr also induced efficient HIV-1 replication in newly infected resting peripheral blood mononuclear cells. Extracellular Vpr transcomplemented a *vpr* mutant virus which was deficient in replication in promonocytic cells, restoring full replication competence. In addition, extracellular Vpr reactivated HIV-1 expression in five latently infected cell lines of T-cell, B-cell, and promonocytic origin which normally express very low levels of HIV RNA and protein, indicating an activation of translational or pretranslational events in the virus life cycle. Together, these results describe a novel pathway governing HIV replication and a potential target for the development of anti-HIV therapeutics.**

Human immunodeficiency virus (HIV) replication is controlled at multiple levels. The interplay of extracellular factors (e.g., cytokines, hormones, activated immune cells, and other pathogens) and intracellular factors (e.g., transcription factors and DNA replication factors) and viral regulatory proteins establish positive and negative regulatory systems controlling virus expression (for reviews, see references 13, 31, 40, 50, 62). The establishment of either productive or nonproductive (latent) HIV infection is regulated both by the virus and by the status of the host cells through poorly defined mechanisms. Latency in HIV infection may be an important mechanism for viral persistence in vivo. The permissiveness of target cells to infection and replication is linked to cellular proliferation, activation, and differentiation, which are in turn regulated by a variety of host factors. For example, T-cell activation is required for the completion of viral reverse transcription and integration (5, 60, 69), though DNA synthesis and cell division are not required for HIV replication in T cells (34, 35). Infected resting T cells may provide a pool of latently infected cells from which virus may be induced after immune stimulation in vivo (3, 70). On the other hand, HIV and lentiviruses in general are unusual retroviruses in their ability to replicate in nonproliferating terminally differentiated macrophages (reviewed in reference 13). Macrophages constitute a second major reservoir for HIV (20, 25, 33, 43) and are often latently infected in vivo (12, 41), and virus replication can be induced by immune activation (41, 54, 55).

* Corresponding author. Mailing address: University of Pennsylvania, 505 BRB1, 422 Curie Dr., Philadelphia, PA 19104. Phone: (215) 662-2352. Fax: (215) 573-9436.

† Present address: Department of Immunology, School of Medicine, Harvard University, Boston, MA 02115.

The *vpr* open reading frame of HIV type 1 (HIV-1) encodes a 96-amino-acid protein with an apparent molecular size of 12 to 15 kDa (66). In vitro studies using *vpr* mutant viruses indicated somewhat slower replication kinetics than those with wild-type virus in T-lymphoid lines and primary T cells (44, 56, 57) and particularly poor replication in primary monocytes (24). Antisense *vpr* phosphorothioate deoxynucleotides have been reported to inhibit HIV-1 replication in macrophages (1). A *vpr-nef* mutant simian immunodeficiency virus (SIV) failed to establish pathogenic infection in monkeys (28), suggesting a vital role in replication in vivo. However the mechanism through which Vpr acts is not known. Vpr may assist Gag functions (37, 47), nuclear localization of the preintegration complex (71), and/or it may assist HIV transcription (7). We have previously reported that *vpr*, alone or in the context of the virus, arrests the proliferation of and induces gross morphological changes in a variety of cell types and allows differentiation of muscle tumor cells (29, and unpublished observations). While wild-type HIV-1 replication was incompatible with rhabdomyosarcoma proliferation, deletion of Vpr from the HIV-1 genome allowed the maintenance of a population of cells which proliferated in the presence of active HIV-1 replication (29), leading to the proposition that through dysregulation of cellular proliferation and differentiation, Vpr may contribute to HIV pathogenesis. Another prediction resulting from that study was that via its effects on cells, Vpr regulates cellular permissiveness to HIV replication.

Vpr protein is found in virions of HIV-1, HIV-2, and SIV (6, 67, 68). The incorporation of Vpr protein into the viral particle provides both a mechanism for delivery of Vpr protein into cells at the time of infection and also a means of export of Vpr from infected cells. Here we report that recombinant Vpr protein, when added to cell culture medium at a low concen-

tration, is a powerful activator of HIV replication in new infection of transformed and primary cells as well as in latently infected cell lines. In latently infected cell lines, Vpr protein increases HIV expression at a translational or pretranslational stage. Further, extracellular Vpr protein establishes a pool of efficient targets of HIV replication, as cells exposed to Vpr protein produce increased virus when infected several days after exposure to Vpr.

## MATERIALS AND METHODS

**Plasmids and cloning: construction of *vpr* baculovirus vector.** A fragment from the pBABE*puro-vpr* vector previously described (29, 30), containing the *vpr* open reading frame of HIV-1 NL43 with the consensus eukaryotic ribosome binding site, was excised using *Bam*H-I and then ligated into *Bam*H-I-cut baculovirus vector pVL1393 (Invitrogen). pVL1393 is derived from the *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) and uses the polyhedrin promoter ($P_{polh}$) for expression of cloned inserts. pVL1393-*vpr* DNA and linearized Ac*M*NPV DNA (BaculoGold, Pharmingen) were transfected into SF9 (*Spodoptera frugiperda*) cells (Invitrogen). To provide control virus not coding for the Vpr protein, pVL1393 vector lacking an insert was transfected along with BaculoGold DNA in a manner identical to that described above and all subsequent expression procedures were carried out as for Vpr expression. Viral stocks used for further infection of insect cells were prepared after plaque purification of recombinant baculoviruses.

**Insect cell culture.** SF9 were grown as adherent cells in Costar T-75 flasks in Grace's modified insect medium (Gibco) supplemented with yeastolate (Gibco)–lactalbumin hydrolysate (Gibco)–10% fetal calf serum (FCS)–penicillin-streptomycin, and fungizone (Sigma) at 25°C. High Five cells (*Trichoplusia ni*) (Invitrogen) were grown in SF-900 medium (Gibco) supplemented as above except without FCS.

**Mammalian cell culture.** TE671 embryonal rhabdomyosarcoma cells (ATCC HTB 139) (39, 61) were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle medium plus 10% FCS–penicillin-streptomycin–sodium pyruvate–25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.4) at 37°C in a humidified 5% $CO_2$ atmosphere. Uninfected H9 (52), SupT-1 (58), and U937 cells and the latently infected lines J1.1 (derived from Jurkat [48]), ACH-2 (derived from an A3.01 clone of CEM [14]), OM.10.1 (derived from HL60 [4]), U1 (derived from U937 [15]), and LL58 (derived from X50.7, an Epstein-Barr virus-transformed B-lymphoblastoid cell line [9]) were obtained from the National Institutes of Health AIDS Reagent Program (NIH ARP). KG-1 was obtained from Georgio Trinchieri. HL60 was purchased from the ATCC. THP-1 was kindly provided by the Medical Research Council AIDS Directed Programme Reagent Project. Peripheral blood mononuclear cells (PBMC) were isolated from a healthy HIV-negative donor by Ficoll-Hypaque centrifugation. All primary and transformed cells were cultured at 37°C in a humidified 5% $CO_2$ atmosphere in RPMI 1640 supplemented with 10% heat-inactivated FCS (or 10% autologous human serum for PBMC)–penicillin-streptomycin–2-mercaptoethanol–25 mM HEPES (pH 7.4).

**Protein expression.** Recombinant baculovirus, either containing (Vpr) or lacking (control) the *vpr* gene, from transfected SF9 cells was used to infect High Five cells at a multiplicity of infection of 5 to 10. High Five cells were grown prior to infection in T-75 flasks and then transferred to a 250-ml spinner flask 1 day before infection and grown as described above. Only healthy (>95% viability) log-phase growth cultures at a cell density of $2 \times 10^6$ cells/ml were infected for protein production. At peak protein production (24 h postinfection), supernatants were collected and centrifuged to remove particulates, and protease inhibitors were added (aprotinin, leupeptin, pepstatin A, each at 2 μg/ml; phenylmethylsulfonyl fluoride, 1 mM; EDTA, 1 mM). Supernatants were then dialyzed against three changes of 100 volumes of 10 mM Tris (pH 8.5) with 10 μM 2-mercaptoethanol (Sigma). Dialyzed supernatants were filtered through a 0.45-μm-pore-size membrane and then supplemented with HEPES (pH 7.4) to 25 mM. Vpr and control supernatants prepared in this manner were stored on ice until use. Vpr and control preparations were mycoplasma and endotoxin negative. No infectious baculovirus remained after these procedures, as determined by an infection plaque assay.

**Preparation of LR1 rabbit anti-Vpr serum.** Recombinant Vpr was purified to about 80% purity by immunoaffinity chromatography using 808 rabbit anti-Vpr serum (obtained from B. Cullen through the NIH ARP [19]), followed by DEAE-Sepharose chromatography. This product was used to immunized a rabbit three times. A fourth and fifth immunizations were performed using three Vpr peptides from the amino and carboxyl termini and the central portion of Vpr (HIV-1 NL43 Vpr amino acids 9 to 20, GPQREPYNYWTL; 41 to 55, SLGQH IYETYGDTWA; and 81 to 96, HFRIGCRSHRIGITRQRRARNGASRS) (purchased from American BioTechnologies) coupled to keyhole limpet hemocyanin. LR1 rabbit anti-Vpr serum recognized recombinant Vpr, Vpr from HIV-1-infected cells, and Vpr in the serum of HIV-positive individuals in enzyme-linked immunosorbent assay (ELISA) and Western immunoblot and did not react with any other cellular or viral proteins. All sera were heat inactivated for use in tissue culture.

**Purification of Vpr by immunoaffinity chromatography.** LR1 immunoglobulin G was coupled to protein G-agarose beads using DMP (22). Dialyzed baculovirus culture supernatant (Vpr containing or control) was passed through the LR1-protein G immunoaffinity column, and then the column was washed extensively with phosphate-buffered saline (PBS) plus 0.5% Triton X-100. Three bed volumes of preelution buffer consisting of 10 mM sodium phosphate (pH 8.0)–0.5% Triton X-100 was passed through, followed by elution buffer consisting of 100 mM triethanolamine (pH 11.5) plus 0.5% Triton X-100. The eluted fractions were collected in 1/20 volume of 1 M sodium phosphate (pH 6.8). For use in tissue culture, Vpr-containing fractions were passed through a detergent removal column (Pierce), dialyzed extensively against PBS, filter sterilized, and stored at 4°C until use. Purified Vpr was used where indicated; alternatively, dialyzed Vpr-containing culture medium was applied at a final concentration of 400 pg/ml. Control column eluate was used as a control for purified Vpr, and control insect culture medium was used as a control when Vpr-containing culture medium was applied. Neither of the negative control preparations (purified protein or baculovirus supernatants) displayed any activity in more than 25 independent experiments. Quantification of purified Vpr was performed by Coomassie staining of sodium dodecyl sulfate (SDS)-polyacrylamide gels, using known amounts of protein molecular weight standards for comparison of band intensity. The Vpr content of baculovirus supernatants was measured by titration in ELISA, using serial dilutions of purified Vpr as a standard. Purified Vpr and Vpr-containing baculovirus supernatants displayed identical activity in HIV replication studies per picogram of Vpr.

**Virus stocks and infection of cells with HIV-1.** Virus stocks were obtained from the NIH ARP, thawed once, aliquoted, and then stored at −80°C until use. An HIV-1 *vpr* deletion mutant, NL43Δvpr, has been previously described (29). For Vpr mutation studies, wild-type NL43 and Vpr mutant NL43Δvpr were produced following transfection of SupT-1 cells by electroporation. Virus stocks were normalized for virus content for infection. Infection of cells was performed by incubating virus with cells under standard tissue culture conditions for 12 h and then washing cells with growth medium to remove unbound virus. Typically, 100 50% tissue culture infective doses ($TCID_{50}$) were used to infect $50 \times 10^3$ to $100 \times 10^3$ cells. No effect on either infection efficiency or Vpr response was observed by using from 10 $TCID_{50}$ to $6 \times 10^4$ $TCID_{50}$. Polybrene had no effect in this assay. No p24 antigen was detectable in supernatants following washing of cells. Culture medium was replaced as required by cell growth, equally to all wells.

**p24 and Vpr antigen capture ELISA.** $p24^{gag}$ antigen was measured by capture ELISA, using a monoclonal antibody in solid phase (V7.8, obtained from Evan Hersh through the NIH ARP) and polyclonal sheep anti-p24 (obtained from Michael Phelan through the NIH ARP) followed by a peroxidase-coupled anti-sheep antibody (Boehringer Mannheim) for detection of bound antigen. Commercial recombinant p24 (ABT) was titrated as a standard curve, and the response was linear on log plots over the range of 0.2 pg/ml to 10 ng/ml. Sensitivity was 2 to 10 pg/ml in tissue culture supernatants owing to a dilution of 1:10 used in the assay. Standard deviation in ELISA duplicates was typically <1% to 10%.

**Fluorescence staining, flow cytometry, and photomicrography.** Prior to being stained, cells were fixed with 80% ethanol–10% acetic acid at −20°C for 10 min and then washed with FACS buffer (Hanks balanced salt solution, 5% horse serum, 0.1% sodium azide, 25 mM HEPES [pH 7.4]). The fixed cells were then incubated with a cocktail of anti-p24 mouse monoclonal antibodies, washed extensively with FACS buffer, incubated with either anti-mouse rhodamine-conjugated antibody (for micrography; Boehringer Mannheim) or anti-mouse fluorescein isothiocyanate (for flow cytometry; Boehringer Mannheim), and then washed extensively with FACS buffer. Cells were fixed with 1% paraformaldehyde immediately after being stained. Immunofluorescence photomicrography was performed using a microscope, color-charged coupled device camera, and video and video printing devices kindly loaned to us by Kristin Kelley and Optical Apparatus, Inc. The original magnification was ×200.

## RESULTS

**Expression of recombinant HIV-1 Vpr.** Native HIV Vpr was produced in insect cells after infection with recombinant baculovirus containing the *vpr* open reading frame cloned from the infectious HIV-1 molecular clone NL43. Surprisingly, the vast majority of Vpr was found in the culture supernatant of recombinant baculovirus-infected insect cells rather than in association with the cells. The recombinant Vpr was identical to Vpr from HIV-infected cells in migration on SDS-polyacrylamide gel electrophoresis (Fig. 1A). Recombinant Vpr had a reactivity identical to that of viral Vpr in Western immunoblot and ELISA with anti-Vpr serum and HIV-positive patient serum. Anti-Vpr serum (LR1) was produced in rabbits after

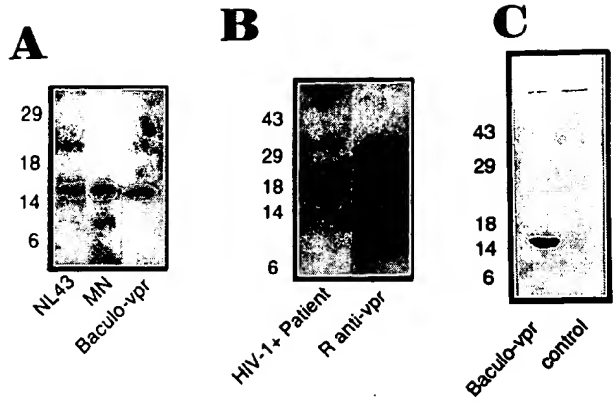



FIG. 1. Recombinant Vpr and reactivity of anti-Vpr antisera. (A) Vpr-containing recombinant baculovirus supernatants and lysates of HIV-1-infected cells (NL43 and MN strains) were run on SDS–15% polyacrylamide gels, transferred for Western blot, and probed with anti-Vpr peptide serum 808. (B) Recombinant Vpr was run and transferred as described for panel A and then probed with either HIV-positive patient serum or with anti-Vpr serum from a recombinant Vpr-immunized rabbit. (C) Purification of recombinant Vpr. Recombinant Vpr was purified by immunoaffinity chromatography, and then 5 μg was loaded on an SDS–15% polyacrylamide gel which was then silver stained. Left lane, eluate from Vpr-containing baculovirus supernatant; right lane, eluate from control baculovirus supernatant.

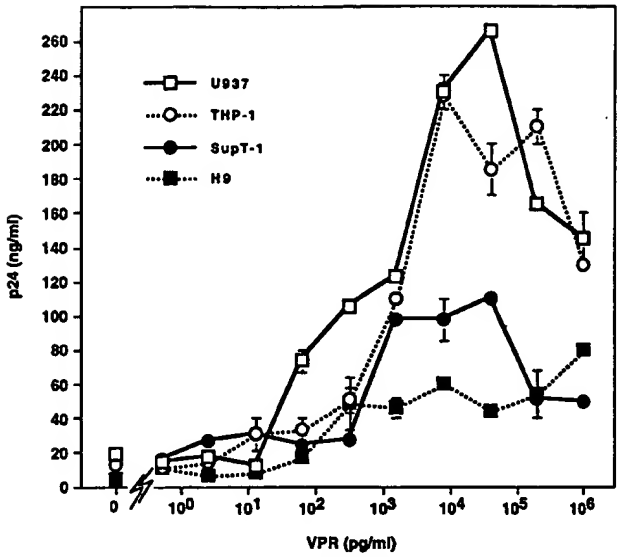



FIG. 2. Dose response of purified recombinant Vpr in HIV-1 infection. The indicated cell lines were infected with HIV-1 in the presence of various amounts of purified recombinant Vpr protein. At 12 h after infection and Vpr exposure, the cells were washed to remove residual virus and Vpr; they were then cultured under standard conditions. $p24^{gag}$ protein was assessed on day 6 by capture ELISA as described in Materials and Methods. p24 values and standard deviations were computed from duplicate cultures.

immunization with partially purified protein and Vpr peptides (Fig. 1B).

**Recombinant Vpr protein displays biological activity.** The presence of most of the Vpr protein in the insect cell supernatant was intriguing and prompted us to investigate the possibility that this protein displayed biological activity as an extracellular molecule. Continuous exposure of TE671 rhabdomyosarcoma cells to Vpr induced growth inhibition and cell differentiation as had transfection with the *vpr* gene (29, 30) (not shown).

We chose to first investigate whether soluble Vpr protein could affect the replication of wild-type HIV, for this might have relevance to infection with wild-type viruses in vivo. Recombinant Vpr protein was purified by using immunoaffinity chromatography (Fig. 1C). Purified recombinant Vpr displayed migration and antibody reactivity identical to that of the crude recombinant protein preparation or native viral Vpr. We exposed four cell lines, two promonocytic lines (THP-1 and U937) and two T-lymphoid lines (H9 and SupT-1), to concentrations of 0.5 pg/ml to 1 μg/ml of purified Vpr at the time of infection with HIV-1 NL43, washed out both the virus and Vpr after 12 h, and then assayed the presence of new virus in the culture medium. Virus production from each cell line was assessed on day 6 by p24 ELISA for each amount of purified Vpr to which the cells were exposed (Fig. 2). The two promonocytic cell lines showed an increase in virus production beginning at less than 100 pg of Vpr per ml for the U937 cell line and around 300 pg/ml for the THP-1 promonocytic cell line. Peak virus production was found using 600 to 2,000 pg of Vpr per ml for each cell line, with an enhancement of 10- to 15-fold in virus production by day 6. Similar data were obtained with two other cell lines, HL60, a promonocytic/erythrocytic line, and the promonocytic line KG-1. The T-lymphocytic cell lines also responded to Vpr treatment with an increase in virus production. A decrease in Vpr response was found with doses greater than $4 \times 10^4$ to $10 \times 10^4$ pg/ml with the exception of the H9 T-lymphoid cell line, which was the least responsive to Vpr. H9 cells continued to show increased virus replication at doses up through 1 μg of Vpr per ml.

Virus production could be detected in the culture medium of the Vpr-treated cells typically 1 day earlier than from the untreated cells and continued to be 10 to 20 times higher for about 7 days after infection (Fig. 3). To further test whether the increase in virus replication was solely an effect of the Vpr protein, U937 and SupT-1 cells were exposed to 320 pg of Vpr per ml at the time of infection in the presence of anti-Vpr antibodies, control antibodies, or medium alone. The Vpr effect was completely inhibited by anti-Vpr antibodies but was not affected by control immune serum (Fig. 3). LR1 anti-Vpr serum also inhibited Vpr activity. After about 1 week following infection and exposure to Vpr, virus production from Vpr-treated and untreated cells was found to be the same, indicating that Vpr functioned to increase the rate of virus production in culture, consistent with previous findings using *vpr* mutant viruses that Vpr increases replication kinetics (24, 44, 56, 57).

**Extracellular Vpr protein complements a *vpr* mutant HIV-1.** We investigated whether extracellular Vpr protein would restore replication to a *vpr* deletion mutant virus. Wild-type NL43 or the *vpr* mutant NL43Δvpr (29) was used to infect SupT-1 or U937 cells in the presence or absence of Vpr. NL43 and NL43Δvpr replicated equally in the T-lymphocytic Sup-T1 cell line, and the extracellular Vpr-treated cells displayed equal increases in virus expression for each virus (Fig. 4). Similar results were obtained with the H9 T-lymphocytic cell line (not shown). On the other hand, NL43Δvpr replicated poorly in the U937 monocytic line. Extracellular Vpr restored the replication of the Vpr mutant in this cell line; in fact the replication of the Vpr mutant plus Vpr was faster in the early days than was that of the wild-type virus without extracellular Vpr but not as fast as that of wild-type virus plus Vpr. Similar results were obtained with the THP-1 monocytic cell line. This result suggests that both endogenously produced (virally encoded) Vpr and exogenous Vpr were active in monocytic infection but that only extracellular Vpr appeared to contribute to HIV replication in the T-cell lines.

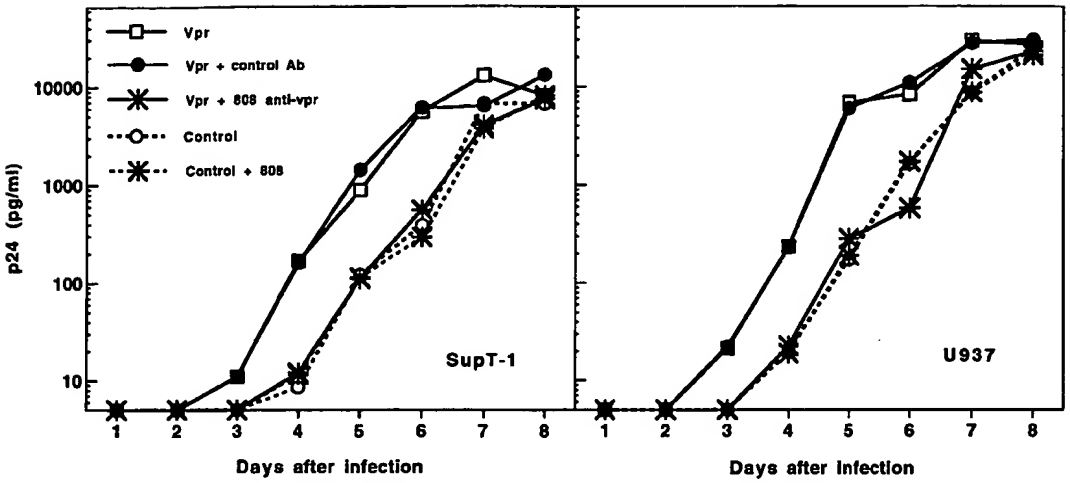



FIG. 3. Time course of HIV infection and antibody inhibition of Vpr activity. SupT-1 T-lymphoblastic cells or U937 monocytoid cells were exposed to 320 pg of purified Vpr per ml at the time of infection with HIV-1 NL43. In addition, some wells received rabbit anti-Vpr serum or an irrelevant rabbit immune serum. Virus production was assessed by p24 ELISA on culture supernatants collected on the indicated days after infection and exposure to agents. p24 values represent mean values from duplicate cultures.

**The Vpr effect is aimed at the cellular targets of infection.** Vpr may directly assist virus replication through an interaction with other viral constituents, or it may regulate cellular events involved in virus replication, or both. The ability of the *vpr* gene to regulate the proliferation and differentiation of various cell types (29) suggested that Vpr may affect cellular permissiveness to productive HIV infection. In order to test this theory, cells were exposed to either Vpr or control protein for 12 h and the cells were then washed and rested in normal culture medium for 5 days prior to infection (Fig. 5). In each case, the Vpr-treated cells displayed an increase in HIV production identical to treatment of the cells at the time of infection. Cells cultured for up to 1 week after Vpr treatment (the longest time tested) retained increased permissiveness to HIV replication. Interestingly, the Vpr mutant virus replicated significantly better in the Vpr-treated monocytic cells than in the control cells, paralleling the results obtained in monocytic cells treated with Vpr at the time of infection.

**Vpr is active in primary hematopoietic cells.** We next examined the activity of extracellular Vpr on natural targets of HIV infection. PBMC from a HIV-negative donor were infected with HIV-1 in the presence of the cell activators phytohemagglutinin (PHA) and interleukin-2 (IL-2) or Vpr or a combination of each for 12 h (Fig. 6); then all agents and infecting virus were washed from the cultures. This transient treatment with PHA and IL-2 failed to increase HIV-1 replication; however, HIV-1 replication was significantly greater in the Vpr-treated PBMC. The combination of Vpr and PHA-IL-2 yielded greater virus production than either alone, indicating that the suboptimal exposure to PHA-IL-2 did affect the cells in a manner that could be enhanced by Vpr.

**Vpr activates virus from latently infected cells.** Cultures of

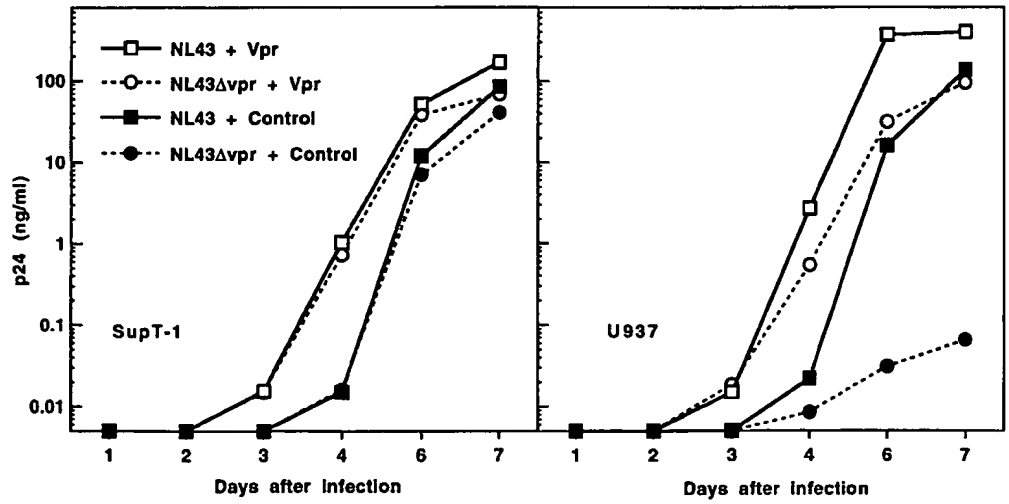



FIG. 4. Extracellular Vpr complementation of a Vpr deletion mutant HIV-1. Cells were infected with either wild-type HIV-1 NL43 or the Vpr mutant HIV-1 NL43Δvpr at the time of exposure to purified Vpr (320 pg/ml). Other procedures were performed as described in the legend to Fig. 3.

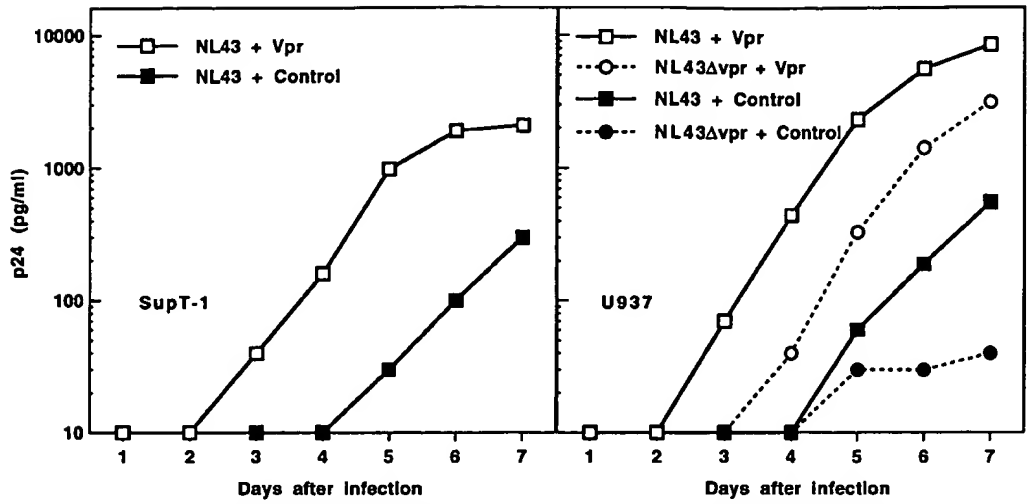



FIG. 5. Vpr induction of a long-lasting permissiveness to HIV replication. Cells were exposed to 320 pg of purified Vpr per ml for 12 h, washed, and then cultured in normal growth medium for 5 days prior to infection. Other procedures were performed as described in the legend to Fig. 3.

cells infected with HIV often spontaneously decline in virus production and enter what is variously termed nonproductive, silent, or latent infection. This tendency of HIV to establish chronic low-level expression may be an important factor in viral persistence in vivo. We next investigated whether Vpr could rescue HIV expression in such cells. A culture of HIV-1-infected THP-1 monocytic cells, from which measurable virus production had declined to undetectable levels 2 weeks after infection, was exposed to soluble Vpr or to the phorbol ester phorbol myristate acetate (PMA) for the first time on day 20 after infection. PMA stimulates HIV transcription through activation of protein kinase C. Virus export resumed after a single exposure to Vpr or PMA, and significant levels of virus production continued for at least 3 weeks following treatment (Fig. 7). The levels of virus replication achieved in the Vpr-stimulated cultures were severalfold greater than those of the PMA-stimulated cultures. Similar reactivation of HIV-1 expression was obtained by using nonproductively infected cultures of U937 and HL60 cells, and in each case Vpr induction was three- to fivefold greater than PMA induction (not shown). In addition, in some experiments, virus export could be measured in the culture supernatant as soon as 14 h after Vpr exposure.

Several well-characterized, latently infected clonal cell lines have been developed as in vitro models of cellular latency (4, 8, 14, 15, 48). These cell lines come from multiple investigators, and with one exception (LL58), each has been shown to resume or increase virus expression after exposure to phorbol ester or tumor necrosis factor alpha. Virus expression has also been induced in the U1 line by IL-6 (49), gamma interferon (49, 59), granulocyte-macrophage colony-stimulating factor

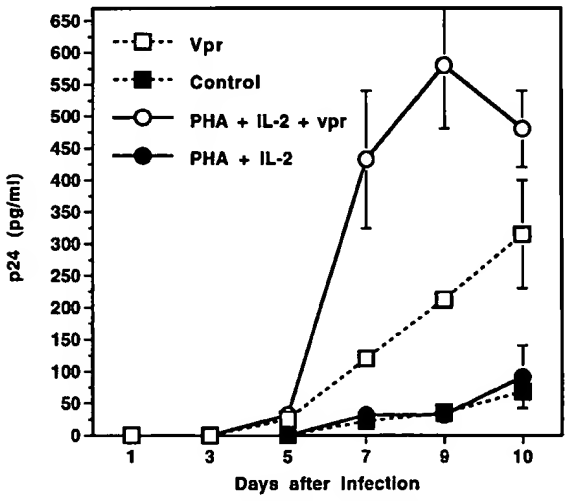



FIG. 6. Vpr increases HIV-1 replication in primary PBMC. PBMC from a healthy HIV-negative donor were prepared by Ficoll-Hypaque centrifugation and then cultured in the presence of the indicated agents for 12 h at the time of infection. PHA, 5 μg/ml; IL-2, 50 U/ml; Vpr, 400 pg/ml. Other procedures were performed as described in the legend to Fig. 3.

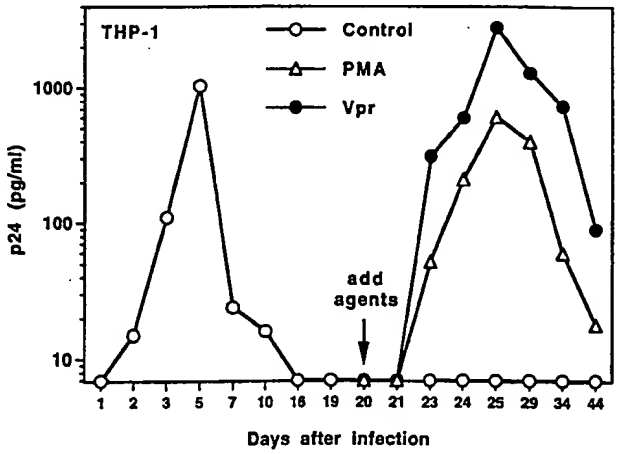



FIG. 7. Reactivation of HIV-1 expression from a spontaneously quiescent culture. THP-1 monocytoid cells were infected with HIV-1 NL43 in normal growth medium and then maintained in normal growth medium (open circles up to day 19). Measurable virus production ceased after day 10. On day 20, Vpr (400 pg/ml) or PMA (50 ng/ml) was added to the cells, which were then cultured in normal growth medium. Vpr, closed circles: PMA, open triangles. Control supernatants had no effect on HIV expression (open circles after day 20).

(15), or heat (59). These cell lines are derived from both T-cell and monocyte lineages, including two parental lines used in the above infection studies (OM.10.1 is derived from HL60 and U1 is derived from U937). The LL58 line is derived from an Epstein-Barr virus-transformed B-lymphocytic line.

Each cell line was exposed to Vpr protein and then examined for virus expression. Intracellular staining for p24 antigen revealed that Vpr reactivated virus protein expression in the cells (Fig. 8). Similar results were obtained with each of the latently infected cell lines. Flow cytometric analysis of p24 staining demonstrates that in all five latently infected cell lines, virus production was reactivated or increased in >90% of the cells (Fig. 9). The two T-cell lines (ACH-2 and J1.1) and one myeloid line (U1) demonstrated constitutive intracellular p24 expression, though extracellular virus was not detected in the culture medium of unstimulated cells. In the LL58 line about 10% of untreated cells were antigen positive, but virus expression was found in >90% of Vpr-treated cells. Virus export was induced in all five cell lines by Vpr (Fig. 10) and was inhibitable with each of the anti-Vpr antibodies (not shown). PMA activated HIV replication in these cell lines to levels similar to that induced by Vpr (not shown).

## DISCUSSION

The results presented here demonstrate that Vpr protein is active as an extracellular molecule acting to increase HIV replication. Purified Vpr protein functioned at very low concentrations, was active on the two main cell types which are targets of HIV infection (T-lymphocytic cells and monocytic cells), but was most active in new infection in monocytic cells, consistent with published Vpr mutation studies. Extracellular Vpr was inhibited by anti-Vpr antibodies. In addition to restoring monocyte replication competence to a Vpr mutant virus, extracellular Vpr boosted the replication kinetics of wild-type HIV-1. Extracellular Vpr rendered cells more permissive to HIV replication, and this highly permissive state lasted several days. In addition to assisting HIV replication in transformed cell lines, extracellular Vpr was active in primary PBMC. Extracellular Vpr reactivated HIV expression in latently infected cell lines which, when uninduced, express no or low levels of HIV RNA and protein.

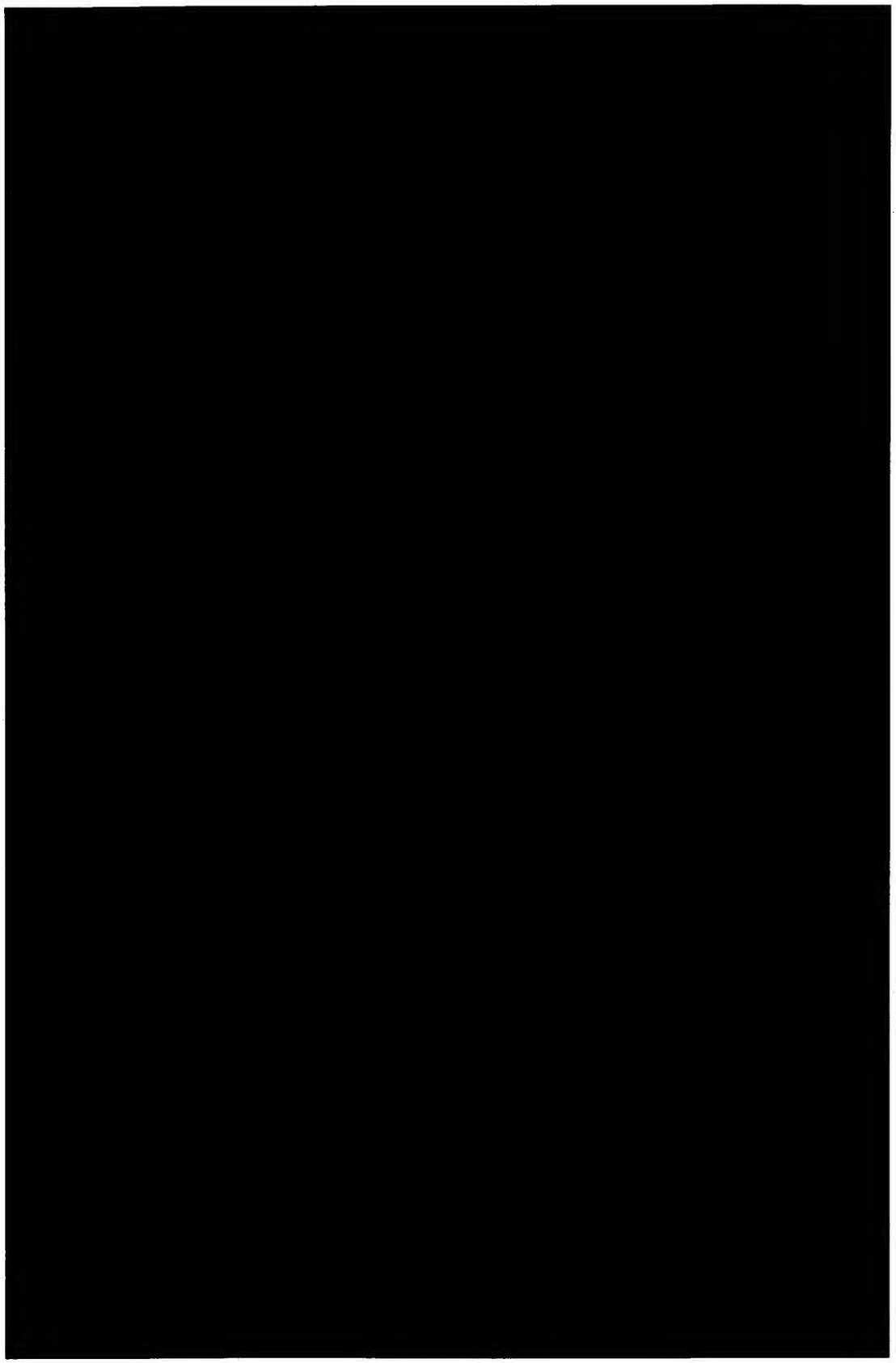
There are numerous precedents for viral proteins with activity as extracellular molecules. Several of these viral proteins mimic or interact with cellular growth factors, oncogenes, or cytokines, each of which influences cell replication or differentiation (reviewed in reference 10). For example, Epstein-Barr virus protein BCRF1 has properties very similar to those of IL-10 and is believed to interact with the immune system and thus promote virus replication and survival (26). Two human retroviral transactivating proteins, HIV-1 Tat and human T-cell leukemia virus type I Tax, are active in soluble, cell-free form (16, 21, 36, 65). Tat has been shown to enter hematopoietic cells and transactivate the HIV long terminal repeat (65). In addition, extracellular Tat can promote cellular growth, indicating a cellular pathway for activity which may be separate from its HIV transactiving function. However, extracellular Tat and Tax are not generally activators of viral replication.

Our previous findings that Vpr, in the context of the virus or when expressed by itself, inhibits the growth of cells and activates the committed cellular differentiation program of muscle tumor cells led to the proposition that Vpr might assist HIV replication via activation of cellular programs which determine cellular permissiveness to virus replication (29). The effects of Vpr on cellular proliferation may be a secondary effect of the regulation of cellular permissiveness, or HIV replication may be more efficient in cells whose growth is specifically arrested. We favor the former interpretation because cellular proliferation generally increases HIV replication. In this report we find a long-lasting alteration in cellular permissiveness to HIV replication. This highly permissive phenotype was induced in both the T-lymphocytic and the monocytic lines. This is a further demonstration that Vpr can act in the absence of other HIV elements, such as Gag proteins, which may be important for additional reported Vpr functions. Vpr may elucidate the expression of cellular factors which assist HIV expression, such as transcription factors, or suppress the expression of cellular factors which would inhibit HIV replication (29). We found that extracellular Vpr increased the replication of HIV in resting PBMC in the absence of other cell stimulators; thus, since Vpr protein did not induce cell proliferation in these cells (data not shown) and the *vpr* gene is cytostatic in several cell types (29, 30), Vpr may alter the cellular phenotype to facilitate replication of HIV in nonproliferating cells.

The similarities between the activities of Vpr protein on latently infected cells and some features of latency in another pathogenic viral system, that of herpes simplex virus (HSV), are striking. HSVs contain within their structures (viral tegument) several virally encoded proteins which appear to be involved in the control of latency (reviewed in reference 18). It has been proposed that the tegument protein brought into cells during infection influences the decision between lytic and latent HSV infection. Tegument proteins ICP0 and Vmw110 are capable of reactivating virus expression in latently infected cells through transcriptional activation of early HSV genes, including themselves, in a probable positive feedback loop (23, 72). In support of this analogy, HIV-2 Vpx protein is structurally and evolutionarily similar to Vpr (42, 63, 64), and virion-associated Vpx has been shown to increase the efficiency of viral replication immediately after infection (27). Thus, HIV may have adopted mechanisms for regulating productive versus latent infection similar to those of classical latent viruses such as HSV.

Recent studies have shown that Vpr may assist early events in HIV infection (71). We have shown here that extracellular Vpr increases virus protein production in latently infected cells, which involves late postintegration events in HIV replication (transcription and translation). The latently infected cell lines ACH-2 and U1 have been found to express very low levels of only fully spliced message prior to induction with cytokines (51). Activation of virus expression requires induction of viral transcription and export of unspliced and singly spliced viral messages coding for the viral structural proteins. Cohen et al. have shown that transfection of Vpr into cells containing reporter constructs can increase transcription from HIV and other promoters by about threefold (7). Our previous findings that Vpr inhibits cell growth and can activate cellular differentiation are also indications that Vpr may affect transcriptional events either directly or indirectly. The Vpr activity

FIG. 8. Activation of virus expression in latently infected cells. (A) Promonocytic line OM.10.1. (B) T-lymphocytic line ACH-2. Cells were exposed to 400 pg of Vpr per ml for 2 days and on day 4 following exposure were stained for intracellular $p24^{gag}$ protein. The left panels were recorded under white light; the right panels show $p24^{gag}$ expression by immunofluorescence. The top two panels of each group of four represent control-treated cells; the bottom two panels of each group show Vpr-treated cells. Untreated cells displayed results identical to those of control-treated cells.



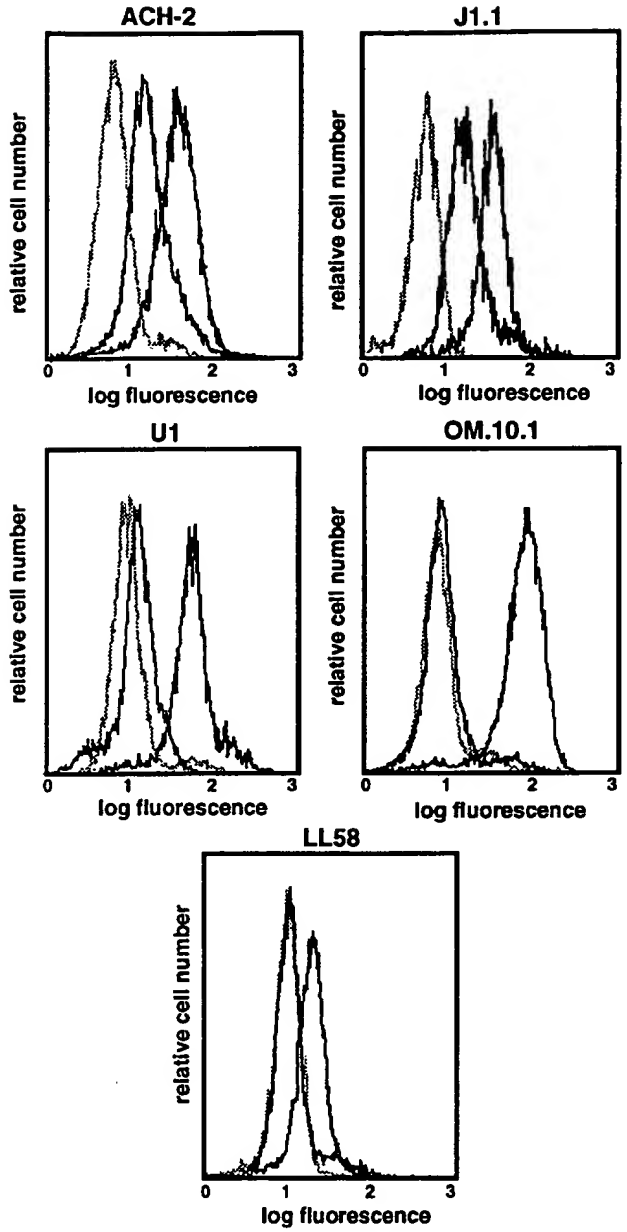



FIG. 9. Flow cytometric analysis of Vpr-treated latent cell lines. Anti-p24 immunofluorescence was performed as described in the legend to Fig. 8 on the latently infected cell lines U1, OM.10.1, ACH-2, J1.1, and LL58. In each histogram, the lightly stippled curve represents background staining with secondary antibody alone. The leftmost black curve in each case represents control-treated cells, and the far right black curve represents Vpr-treated cells. Untreated cells presented staining identical to that of control-treated cells.

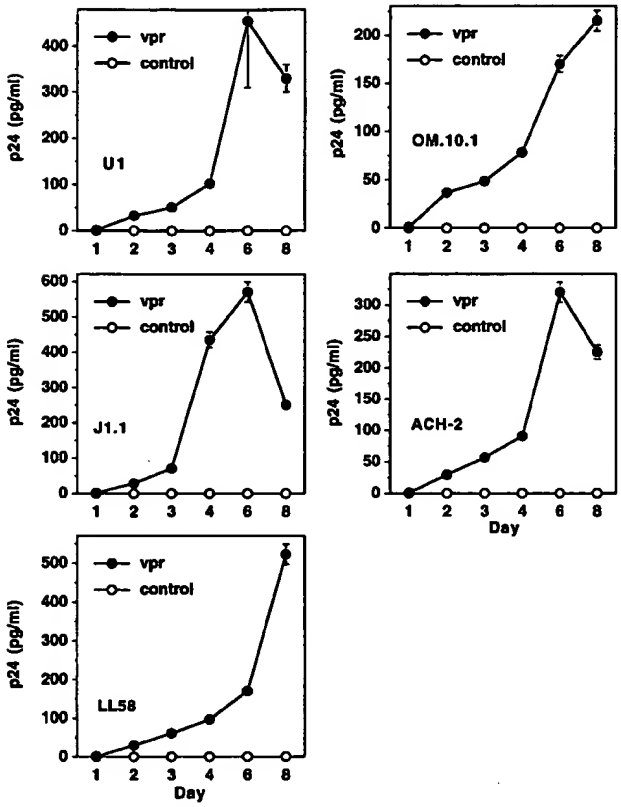



FIG. 10. Rescue of virus expression from latently infected clonal T lymphocytes, B lymphocytes, and monocyte lines. Five latently infected cell lines were exposed to 400 pg of Vpr per ml on day zero. p24 content of the culture medium was assessed as described in the legend to Fig. 3. Standard deviations were computed from duplicate cultures.

reported here might explain transcellular activation of the HIV long terminal repeat observed after coculture of HIV-infected cells with target cells containing reporter constructs (38). The experiments reported here do not indicate whether Vpr assists early or late HIV replication events in newly infected cells. However, since Vpr assists late events in cells which are already (latently) infected, we prefer the notion that extracellular Vpr activates late events in newly infected cells as well. This concept has the advantage of simplicity plus the added attraction of unifying the pathway for Vpr enhancement of HIV expression in latently and productively infected cells through a regulation of cellular permissiveness to HIV replication.

The long-lasting increase in cell permissiveness reported here indicates that Vpr may create a "pool" of highly permissive potential targets of infection. Since Vpr is specifically incorporated into the viral particle in amounts similar to those of the Gag proteins (6), Vpr is exported from infected cells at a high level. Virus disintegration or immune lysis of virions could release Vpr into bodily fluids where autocrine or paracrine regulation of HIV replication would ensue. We have recently found biologically active Vpr in the serum and cerebral spinal fluid of HIV-infected individuals in levels that correlate with the degree of p24 antigenemia observed and disease state (30a). The high level of virus replication that occurs after initial infection, and also at the last stage of disease, may be accelerated by a positive feedback mechanism driven by free extracellular Vpr. Extracellular Vpr could also provide a means to reactivate virus expression in latently infected cells in vivo. The majority of infected peripheral blood lymphocytes do not express measurable amounts of viral antigens and must be stimulated in vitro with mitogens or other activators in order to express virus (2, 3, 17, 32, 53). The percentage of infected cells in the lymphoid tissues is much greater than that in the peripheral circulation (45, 46), and recently it has been reported that large amounts of latently infected cells persist in these tissues throughout the course of HIV infection (11, 12, 46).

The ability of Vpr to activate both new virus expression and virus export in all latently infected cell lines tested suggests that Vpr could activate virus expression in areas, such as lymphoid tissues, in which high local concentrations of viral antigens are found. Since Vpr also regulates the proliferation and development of diverse cell types, extracellular Vpr might contribute to tissue-specific pathologies associated with HIV infection, including wasting, neurological disease, and T-cell depletion.

## ACKNOWLEDGMENTS

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72. *NIAID Council News* 1, 3 (1992). The NIAID HIV Vaccine Working Group is a collaboration between government and nongovernment scientists and community representatives that will guide HIV vaccine research and development. The HIV Vaccine Working Group will begin to centrally coordinate the efforts of the National Cooperative Vaccine Development Group of NIAID, academic and industry scientists, and community and patient advocacy groups. Once an HIV vaccine candidate is available that satisfies many or all of the criteria of a successful preventive vaccine, then an even more directed project may be necessary to facilitate resolution of patent conflicts and to resolve liability and other logistic problems that might prevent implementation of an effective HIV preventive vaccine. The co-chairs of the HIV Vaccine Working Group are D. Bolognesi and D. Hoth. Current participants in the working group are G. Ada, A. Ammann, A. Carney, L. Corey, R. Desrosiers, J. Dickson III, B. Graham, B. Haynes, M. Hilleman, D. Ho, D. Hodel, H. Jaffe, N. Letvin, H. Temin, R. Vasquez, and S. Zolla-Pazner. NIH staff on the committee are D. Alexander, L. Barker, W. Blattner, J. Bradac, P. Fast, R. Hoff, M. Johnston, H. C. Lane, B. Mathieson, P. Pizzo, A. Schultz, S. Vermund, F. Vogel, and J. Whitescarver.
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# Present Status and Future Prospects for HIV Therapies

Margaret I. Johnston* and Daniel F. Hoth

**Since the discovery of human immunodeficiency virus (HIV) in 1983, significant progress has been made toward the discovery, development, and licensing of anti-HIV drugs. In vitro screens against whole virus are now being complemented by screens against specific viral targets, resulting in the development of clinical candidates acting at several critical stages of the viral life cycle. Despite these advances, clinical therapy remains largely palliative. In addition, it has recently been recognized that HIV resistance to most drugs may pose even greater obstacles. Moreover, emerging data on immunopathogenesis raise the possibility that even if virus was eliminated from an infected individual, the patient's immune system might not be capable of restoration to normal function. In the face of such obstacles, deeper insights into the pathogenic mechanisms of disease, aggressive exploitation of those mechanisms for therapeutic gain, and continued commitment of both public and private sectors to support and collaborate in this research are needed.**

## Introduction

In 1983, when HIV was discovered, the only antiviral agents licensed in the United States were amantadine, vidarabine, and acyclovir (*1*). Research was initially slow because only a limited number of facilities were willing to handle HIV, a new, lethal infectious agent. Fortunately, a significant body of information on the genomic structure and replication cycle of retroviruses had accumulated over the previous two decades (*2*) (Fig. 1).

Nucleoside analogs were a logical first place to search for anti-HIV agents because reverse transcriptase (RT) catalyzes a reaction not known to occur in humans and because several companies had libraries of nucleoside analogs synthesized in the search for anticancer or antiviral agents. In 1984, 3′-azidothymidine (AZT) was identified as active, first against murine retroviruses and then against HIV in cell culture (*3*). Clinical testing began in 1985. The phase II trial that conclusively showed a survival advantage for individuals with advanced disease taking AZT versus placebo was completed in September 1986, only 3 years after identification of HIV. The speed at which AZT was discovered, moved through clinical trials, and approved was unprecedented. Recognition that AZT did not completely suppress disease and had associated toxicities served as a stimulus for expanded research to identify additional agents.

The first inhibitors of HIV replication were discovered as a result of cell culture–based screening efforts, and such efforts continue to be valuable in identifying new agents that act at any step in the viral replication cycle. Recombinant DNA technology made possible the eventual cloning

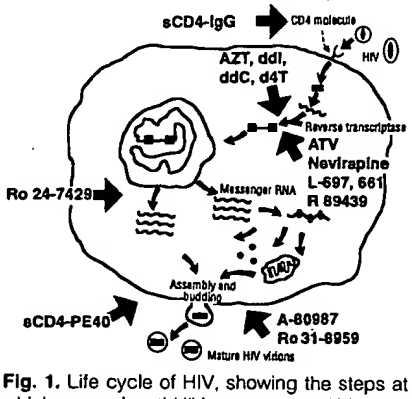


**Fig. 1.** Life cycle of HIV, showing the steps at which several anti-HIV agents act. Abbreviations are explained in the text.

M. I. Johnston is the associate director, Basic Research and Development Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, MD 20892. D. F. Hoth is the director, Division of AIDS, NIAID, NIH, Bethesda, MD 20892.

*To whom correspondence should be addressed.

of several key HIV proteins, which in turn stimulated development of mechanism-based screens that could accommodate large numbers of agents rapidly, inexpensively, and safely. Only recently have products identified by mechanism-based screens and confirmed in cell culture assays advanced to clinical trial. Structure-based primary screening activities have also been described, and although novel inhibitors have been identified, none has yet demonstrated sufficient activity against HIV in cell culture assays at nontoxic concentrations to warrant further development.

Typically, a clinical candidate is selected because of demonstrated potency in vitro at concentrations (usually micromolar or lower) that are anticipated to be maintained in the bloodstream or intracellularly for several hours and which are significantly lower than concentrations toxic to cells or animals. Several agents have shown potent activity in cell culture assays but have not been considered for further development because pharmacologically active blood levels could not be maintained or tolerated (*4–6*). Because both $CD4^+$ T cells and monocytes-macrophages are infected in the body, agents that show activity in both cell types are favored. In addition, because cells in the central nervous system (CNS) can be infected with HIV, the ability to penetrate the blood-brain barrier is considered. Finally, because almost all are virustatic and must be taken over a prolonged period, orally administered agents are preferable.

This article will focus on therapies for HIV infection and immune restoration. Although opportunistic infections (OIs) arising late in the course of disease secondary to severe immunosuppression are the principal cause of morbidity, discussion of this diverse group of diseases is beyond the scope of this article. Furthermore, unless interventions that result in fundamental improvements in immune function or reduction in viral replication are developed, effective treatment of one OI will only permit emergence of yet another.

## Current State-of-the-Art Treatment

Although it is difficult to prove, treatment of HIV has likely resulted in prolonging the life of HIV-infected individuals and improving their quality of life. There are currently three antiretrovirals approved for use in HIV disease: 3′-azidothymidine (AZT, zidovudine, Retrovir), 2′,3′-dideoxyinosine (ddI, didanosine, Videx), and 2′,3′-dideoxycytidine (DDC, zalcitabine, HIVID). Numerous others are in clinical trial.

Approval of AZT was based on a placebo-controlled trial in individuals with advanced acquired immunodeficiency syndrome (AIDS) (<200 $CD4^+$ cells per cubic millimeter); the trial demonstrated a significant difference in mortality in the drug and placebo groups (*7*). Subsequently, AZT administered earlier in disease (<500 $CD4^+$ cells per cubic millimeter) was shown to delay the onset of AIDS-associated OIs (*8*). Furthermore, a recent preliminary report of a European trial of AZT versus placebo in asymptomatic HIV-infected persons suggests that the duration of AZT benefit may be limited, because there was no difference in survival at 3 years (*9*). Additional trials of AZT in asymptomatic HIV-infected persons are in progress.

Although ddI, another potent inhibitor of RT, produced a moderate rise in CD4 cell number, it was not shown to be superior to AZT as initial therapy (*10, 11*). Dideoxyinosine is approved for use in individuals who have been on AZT for 4 months or longer or who are hematologically intolerant to AZT. The combination of AZT and ddI has shown promising results in phase I evaluation (*12*). DDC, on a molar basis, is the most potent of the three approved drugs in cell culture assays (*13, 14*) and has been approved for use with AZT in adults with <300 $CD4^+$ cells per cubic millimeter who have experienced significant clinical or immunologic deterioration (*15*). Additional efficacy trials of AZT, ddI, and DDC are in progress.

Antiretroviral therapy is routinely used in the United States to treat individuals with moderate to advanced disease (for example, <500 $CD4^+$ cells per cubic millimeter). Experimental monotherapies are typically evaluated for their ability to delay progression to disease; determining the clinical impact of new therapies on survival has been problematic. Although the first randomized trial with AZT was placebo-controlled and demonstrated a survival advantage, subsequent trials have used active controls, and survival gains over AZT have not been shown. However, it is possible that the therapies evaluated to date have an equivalent effect on survival, so that such trial designs may not be capable of detecting mortality impact. Even drug-induced delay of disease progression is a problematic end point because trials among patients with early disease may require years before sufficient clinical end points accumulate. This has led to the search for laboratory tests, such as measurement of $CD4^+$ cells, or quantitative measurement by polymerase chain reaction (PCR) of circulating virus that could be used as surrogates and that could yield evidence of a drug effect more quickly and with greater sensitivity. Development of reliable assays and validation of these markers remain a challenge (*16*).

Since late 1989 there has been a reduction in the quarterly incidence of AIDS, that is, in the transition from the asymptomatic to the symptomatic state, as compared with the predicted AIDS rates (*17*). This result cannot be fully explained by a reduced rate of infection. The change in rate of progression to disease is probably associated with the widespread introduction of AZT into the population (as well as with prophylaxis for *Pneumocystis carinii* pneumonia) and is confined to those populations with more ready access to adequate health care.

*Why are licensed RT inhibitors not entirely successful?* The failure of existing therapies to completely block clinical progression remains one of most important questions facing therapeutic researchers. AZT and ddI, at clinically used doses, reduce circulating viral burden by only about one-half to one-tenth of initial values (*18, 19*). Although an RT inhibitor would not be anticipated to decrease the production of virus from cells already infected with HIV, effective concentrations should protect new cells from becoming infected. However, individuals do progress to disease while on antiretrovirals. Further, changes in the genomic sequences of HIV circulating in patients suggest that active infection of new cells and the error-prone process of reverse transcription occurs.

One possible explanation for the failure of existing therapies to halt progression is a failure to maintain adequate drug levels at the site of viral replication over extended periods. Current anti-HIV therapies, once begun, require frequent administration of drug owing to the relatively short half-lives of these drugs and are usually continued throughout life. Hence, drug compliance remains a major issue, and drug failure may be due to the inability to maintain drug concentrations at adequate levels. In particular, drug concentration in infected tissues and especially in intracellular compartments remains poorly studied. For example, for certain nucleosides [ddI, DDC, and the (−) enantiomer of 2′-deoxy-3′-thiacytidine (3TC, Lamivadine) (*20*)] the ratio of active nucleoside triphosphate to natural substrate is higher in resting than activated cells, whereas for others [AZT and didehydrothymidine (d4T, Stavudine)] the opposite is found (*21*). The impact of cell activation on the intracellular levels of other anti-HIV agents remains unknown.

Another possible explanation for drug failure is the emergence of drug resistance. Resistant virus can be isolated in cell culture and from individuals after about 6 months to 1 year of AZT treatment; resistance appears to become more frequent in later stage patients (*22*). Resistance may arise more quickly in patients with more advanced disease because they have a higher and more genetically diverse viral burden and are thus more likely to have preexisting resistant species that become selected in the

presence of drug. However, it has not been proven that the emergence of drug-resistant phenotypes, as measured in cell culture assays, is associated with clinical deterioration, although correlations have been suggested (*23, 24*).

Resistance has been described for each of the widely used antiretroviral nucleosides. The molecular basis for resistance to AZT inhibition is associated with mutations at at least four key loci of the HIV RT, with multiple mutations resulting in the highest degree of resistance (*25*). In addition, certain loci are more critical than others in maintaining drug susceptibility (*26*). Mutations associated with resistance to ddI and DDC have also been found in clinical isolates (*27*). There are now troublesome reports of possible transmission of AZT-resistant phenotypes (*23, 28*). Determining the extent and significance of such transmission is a public health priority.

Mutations associated with resistance, although seemingly counter to successful therapy, could have a beneficial outcome. Certain combinations of mutations linked with drug-resistant phenotypes might yield nonfunctional RT and noninfectious HIV (*29*). Four mutations associated with resistance to three different drugs (AZT, ddI, and a nonnucleoside RT inhibitor) were engineered into HIV and shown to result in replication-incompetent virus. Exposure of chronically infected cells to the above drug combination, and passage of the infected cells for several weeks, first in the presence of the drug combination and then in its absence, resulted in a sterile culture. However, it has not yet been demonstrated that exposure of HIV to drug combinations in cell culture resulted in selection of genotypic mutations associated with multidrug-resistant, replication-incompetent phenotype. In addition, other combinations of AZT, ddI, and nonnucleoside RT inhibitor (NNRTI)–induced mutations were consistent with HIV replication (*30*). Furthermore, this was not the first report of a drug-induced sterile culture, which could be the result of complete inhibition of HIV replication coupled with death or diluting out of infected cells (*31, 32*). Regardless, the combination of AZT, ddI, and nonnucleoside inhibitor was highly potent. A definitive clinical trial of this combination is in progress.

Other approaches attempting to overcome or prevent selection for drug resistance include, first, the use of drug-resistant HIV, particularly resistant clinical isolates, in screens for new drugs. Second, early treatment before significant numbers of mutants are generated may be worthwhile if resistance is the result of selection of preexisting mutants and given current data suggesting that initial infection is usually established by a single genotype. Two studies are under way to evaluate AZT treatment initiated within weeks of infection. Third, the benefit of switching patients onto a different antiretroviral therapy when evidence of a resistant genotype first appears is currently being evaluated.

## Therapies in Development

*Other RT inhibitors.* The successful results obtained with AZT stimulated researchers to find additional RT inhibitors, many of which are now undergoing clinical evaluation. Use of d4T has resulted in increased $CD4^+$ cell counts and a decline in p24 antigenemia in previous trials and is currently being evaluated versus AZT use in phase II and phase III clinical trials in HIV-infected individuals with at least 6 months of prior AZT therapy (*33*). Toxicities associated with higher doses of d4T have included peripheral neuropathy and hepatitis. Another nucleoside, 3TC, is currently in phase I and phase II clinical trial; preliminary reports cite a transient increase in $CD4^+$ cell counts and decline in serum p24 levels, although no clear dose-response relation has been observed (*34*). No significant toxicity has been noted at the doses evaluated. On the basis of cell culture data, 3′-fluoro-thymidine (FLT) is among the most potent of the 3′-halo-dideoxypyridine analogs reported to date (*35, 36*). Preliminary clinical results suggested some activity in vivo (*37*). Further development of FLT has recently been discontinued, suggesting that significant antiviral activity was not observed at plasma concentrations that were well tolerated in a recent controlled phase II study. 9-(2-Phosphonomethoxyethyl)-adenine (PMEA), an acyclic nucleotide, recently entered phase I clinical trial (*38*). PMEA was more effective than AZT in blocking simian immunodeficiency virus (SIV) infection of macaques under optimal conditions of drug administration before infection (*39*). Clinical development of PMEA should be undertaken with caution in view of the narrow therapeutic range observed in cultured cells and in studies with mice demonstrating significant side effects, including fetal resorption and death of pregnant female mice (*40*). Other nucleosides are at earlier stages of development.

The development of a nonnucleoside inhibitor of HIV RT and replication was first reported in 1990; subsequently, other classes of RT inhibitors were reported, including tetrahydro-imidazo[4,5,1-jk][1,4]-benzodiazepin-2(1H)-one (TIBO, R82913) (*41, 42*), 11-cyclopropyl-7-methyl-dipyrido-[2,3-b:3′3′-f]1,4-diazepin-6H-5-one (BI-RG-587, nevirapine) (*43*), pyridones (L-697,661 and L-696,229) (*44*), and bis(heteroaryl)piperazines (BHAPs, U-87201E, Atevirdine Mesylate, ATV) (*45*). Two additional agents in this class, R-89439 [an α-anilino-phenylacetamide (α-APA) derivative (*46*)] and a second generation BHAP U-90,152 (*47*), recently entered clinical trial.

Although structurally distinct, NNRTIs have several features in common. All are extremely potent in cell culture assays and inhibit HIV replication at nanomolar concentrations (*41–49*). Unlike AZT, ddI, or DDC, the NNRTIs do not require conversion to active drug once within the cell. These agents typically have therapeutic indices defined in cultured cells in excess of 1000. They are highly specific noncompetitive inhibitors of HIV and do not inhibit other retroviruses, including the closely related SIV and HIV-2. Most appear to bind to a site on RT near $Tyr^{181}$ and distinct from the substrate binding site (*48–51*). Decreased sensitivity of HIV to these agents develops rapidly, both in cultured cells and in vivo (*31, 47, 50–53*). HIV resistance to one NNRTI is usually cross-resistant to the other classes of NNRTIs, with the possible exception of the BHAPs class of RT inhibitors (*31, 47, 50, 51*).

In phase I trials, NNRTIs produce a rapid but transient decline in serum p24 levels (*54*). Virus with drug-resistant phenotype can be isolated within weeks after the onset of treatment. The favorable oral bioavailability of the NNRTIs and the lack of significant toxicities of these agents have stimulated a search for more potent NNRTIs that might overcome resistance. To date, NNRTIs appear to be additive or synergistic with nucleosides (*55*), and several combinations are currently being, or will soon be, evaluated in clinical trials in the hope that highly resistant isolates will not emerge. In addition to agents that block HIV replication at the RT stage, therapeutics that act at other stages are being sought. These will be discussed in an order that reflects the relative priorities currently being accorded to their development.

*Protease inhibitors.* HIV protease cleaves polyprotein precursors into mature structural proteins and enzymes during particle assembly and maturation (*56*). Although genetically engineered virus containing mutated protease that lacks enzymatic activity replicates, the virions that are produced are noninfectious in cultured cells (*57*). Protease inhibitors are an attractive target for therapeutic intervention because they act at a postintegration step of HIV replication. Recent cell culture data suggesting that cell-associated virus is more infectious than free virus magnify the need to evaluate agents that block the spread of HIV from infected cells (*58*). Whereas RT inhibitors are only effective in blocking HIV replication when added to cultured cells before HIV infection, protease inhibitors can also

inhibit HIV production from chronically infected cells (59, 60). Additional factors combine to make the HIV protease an attractive target for therapeutic intervention, including its unique cleavage specificity relative to human aspartic proteases, availability of cloned and chemically synthesized protease, detailed structure information, and the availability of rapid protease assays (61).

Peptide-based substrate analogs were the first inhibitors of protease reported to be active in vitro (59, 60, 62) and were the first to enter clinical trial. Development of peptide-based inhibitors has presented several challenges. In general, attempts to synthesize many of these inhibitors have required multistep, low-yield efforts. Modifications were needed to protect the peptide from degradation, while maintaining bioavailability, solubility, and activity. The first protease inhibitor to enter clinical trial, Ro 31-8959 [reported by Roberts (59, 60)], was an orally administered hydroxyethylamine mimetic of the transition state and had fairly low solubility and low bioavailability (62). Finally, development of protease-resistant HIV in cell culture has been reported, although the level of resistance has been low (10- to 30-fold) and cross-resistance is usually not complete (63). Thus, resistance to protease drugs may not prove to be as problematic as NNRTI resistance, which can be 1000-fold (31, 47, 50–53).

Recent studies by Kempf (59, 60) on the C-2 symmetric protease inhibitor A-77003 [originally designed to fit the C-2 symmetric protease active site (64)] led to identification of a second generation inhibitor, A-80987, with improved oral bioavailability and serum half-life in animals (65). A-80987 is currently in phase I trials in Europe. Other protease inhibitors, including several that will probably be orally bioavailable, are at earlier stages of development and will probably soon enter clinical trial. Protease inhibitors tested are additive or synergistic with AZT, ddI, or DDC, which suggests that combinations of therapies directed to different drug targets will prove to be useful (66).

*Tat inhibitors.* Tat, a regulatory protein required for HIV replication in cultured cells, is a positive transactivator that stimulates transcription (67) and that may have other activities (68). An anti-Tat agent capable of blocking HIV replication in both acutely and chronically HIV-infected cells, 7-chloro-5-(2-pyrryl)-3H-1,4-benzodiazepin-2(H)-one (Ro 5-3335), was first reported in 1991 (69). A less toxic clinical candidate, Ro 24-7429, entered clinical trial in 1992. A multisite trial is under way to study safety and to determine the impact on viral load and $CD4^+$ cell count. Although Tat-resistant HIV has not been observed even after 2 years of virus passage in cell culture under conditions similar to those used to generate resistance to other anti-HIV therapies (70), examination of clinical isolates will be needed. The clinical usefulness of anti-Tat agents in combination with anti-RT agents should be investigated.

*Blocking of viral entry.* HIV entry begins with highly specific binding of the HIV gp120 envelope protein with a CD4 molecule on the surface of most susceptible cells. In addition, binding of gp120 on the surface of an infected cell with CD4 on the surface of an uninfected cell is involved in syncytia formation and cell-to-cell spread of HIV. A recombinant soluble form of the CD4 receptor (sCD4) or the chimeric CD4–immunoglobulin G (IgG), designed to extend the serum half-life of sCD4, effectively blocked HIV infection and syncytia formation in cultured cells at levels that were attainable clinically (4, 71). However, in initial clinical studies viral markers were not affected (5). Primary isolate virions were shown to have a significantly decreased ability to bind sCD4 and were less sensitive to neutralization by sCD4 in vitro as compared with cell-cultured adapted isolates (72). Higher doses of sCD4 may be tolerated without toxicity, but it may not be practical to pursue such studies.

Another agent designed to exploit the interaction of CD4 and gp120 is CD4-PE40, a fusion protein between CD4 and two domains of the *Pseudomonas aeruginosa* exotoxin A (32, 73). CD4-PE40 binds to infected cells through interaction with gp120 expressed on the cell surface. One toxin domain facilitates entry of the lethal second domain into the cell, resulting in death of infected cells in culture. Phase I trials demonstrated dose-limiting hepatotoxicity. Because CD4-PE40 and AZT synergize in vitro, clinical studies are in progress to determine whether any combination of dose and schedule has a beneficial effect without unacceptable toxicity. This strategy represents one of the few virucidal approaches to the treatment of HIV. However, toxicity may eventually prove to limit the clinical usefulness of this approach.

*Other HIV targets.* HIV RT has three distinct enzymatic functions: (i) the polymerase domain, which catalyzes the transfer of nucleotides onto the growing DNA chain, is the primary target for existing nucleoside and nonnucleoside inhibitors of HIV RT; (ii) a ribonuclease (RNase) H domain cleaves genomic RNA after first strand synthesis to allow synthesis of the second strand DNA; and (iii) a double-stranded RNA-dependent RNase cleaves in the primer-template pair at specific sites (74). RNase H is required for HIV replication (75). Only a few agents have been reported to target RNase H (76, 77). 3′-Azidothymidine monophosphate, which accumulates to millimolar levels in cells treated with AZT, inhibits RNase at millimolar levels; RNase inhibitors may be a secondary mechanism by which AZT inhibits its RT activity (76, 78).

Two other HIV-encoded proteins are being examined as possible targets for therapeutic intervention. Integrase, an enzyme characteristic of retroviral infection, is essential to replication (79, 80). A rapid microtiter assay for the joining activity catalyzed by HIV integrase has been described (81), although no inhibitors of integrase have been reported to date. Rev permits the export of unspliced HIV mRNAs from the nucleus and is also required for HIV replication (82). Recently, a high-throughput screen based on Rev-dependent expression of gp160 in *Drosophila* cells has been established (83).

A cellular myristoylCoA:N-myristoyltransferase (NMT) catalyzes transfer of myristate from myristoylCoA to the HIV-encoded proteins Nef and Gag in a process required for HIV replication. Heteroatom-containing analogs of myristic acid, such as 4-oxatetradecanoic acid, serve as substrates for NMT (84, 85). Addition of these analogs to HIV Gag results in alteration of protein hydrophobicity and localization of Gag in the cytosol rather than the plasma membrane. Inhibition of HIV replication in cultured cells can be achieved with these analogs at concentrations that are not detectably toxic to uninfected cells (85, 86). It remains to be seen whether addition of myristic acid analog will be sufficiently restricted to viral rather than cellular proteins to provide an acceptable therapeutic window. Finally, a number of natural products whose mechanism of action may or may not be known are at various stages of development (87).

*Nucleic acid–based therapies.* Nucleic acid–based therapeutics, many of which target virally encoded nucleic acids, offer unique opportunities for intervention, but remain wholly unproven. Nucleic acid–based therapies being evaluated in cell culture include antisense oligonucleotides (88, 89), catalytic RNAs or ribozymes (90, 91), RNA analogs or decoys (92), and genes that encode proteins, such as CD4 or trans-dominant peptides, that have direct antiviral activity (93).

Antisense oligonucleotides directed against sites proximal to and including the translation initiation codon, splice sites, and single-strand loops were reported to be successful in blocking HIV replication in cultured cells at micromolar levels (94, 95). The use of catalytic RNAs or ribozymes that recognize and cleave specific viral sequences in trans has been proposed as an

approach to decrease the amount of oligonucleotide needed to target a specific intracellular RNA (*91*). Cultured cells stably transfected to express a ribozyme gene targeted against the HIV *gag* mRNA were shown to be partially resistant to HIV infection; cleavage of *gag* mRNA in the predicted location was demonstrated (*96*). In theory, catalytic RNAs can inactivate many target RNA molecules within the same cell, although turnover of catalytic RNA in intact cells has not yet been demonstrated. Oligonucleotides, such as TAR decoys, polyTAR, and RRE decoys, that inhibit the function of viral proteins are also under investigation (*92*).

Exogenous delivery of oligonucleotides has been plagued by problems of nonspecific inhibition and toxicity, inefficient cellular uptake, and instability in plasma. Stability issues have been addressed to some extent through chemical modifications of the oligonucleotide or, in the case of ribozymes, through DNA-RNA chimeras. Liposome or lipofectin encapsulation or lipophilic modification increases the efficiency of uptake of nucleic acids into cultured cells (*97*). A critical step in exogenous delivery of nucleic acid therapies will be to assure that the delivered material escapes the lysosomal degradation pathway after internalization into the cell. Despite the difficulty of obtaining sufficient quantities of nucleic acid to administer systematically, clinical trials are likely to occur within the next year as several antisense and ribozyme approaches are developed further.

Recent advances in the application of gene therapy to several diseases have stimulated interest in the therapeutic potential of nucleic acids expressed endogenously by cells. Although several approaches have succeeded in producing anti-HIV activity in cultured cells, the difficulty in obtaining sufficient expression of the desired gene in a sufficient number of cells in vivo has remained an obstacle. Currently, transfection with retroviral vectors results in expression of the desired gene in only a small percentage of cells. Other methods of delivery have been proposed, including a retroviral vector that makes use of the highly efficient HIV long terminal repeat (LTR) to control gene expression (*98*). Although HIV vectors have the advantage of infecting the same target cell as HIV, there may be serious drawbacks associated with their use. Approaches are needed to ensure that these vectors are devoid of pathogenic capability and to eliminate the risk of recombination or mutation. Adeno-associated virus vectors that can infect diverse cell types with higher efficiency have also been described but are not yet available for clinic use (*99*). Another approach is the use of adenovirus capsids that bind the gene to be delivered through an antibody-polylysine complex attached to the capsid (*100*). Capsids may be readily formulated with the nucleic acid to be delivered and may deliver large amounts of nucleic acid into the cell, although gene expression is transient and the efficiency of T cell transduction is low.

*Immune reconstitution.* Approaches to block HIV replication are complemented by approaches to manipulate the immune system. The use of candidate HIV vaccines to increase existing immune responses or stimulate new ones in HIV-infected individuals is reviewed elsewhere (*101*). Another immunization-based approach is the ex vivo retrovirally mediated introduction of the *env* gene into autologous fibroblasts (*102*), which would then be given back to the patient to stimulate anti-*env* immune responses. Studies of cytotoxic T lymphocytes (CTLs) generated by immunization of mice with syngeneic cells expressing HIV (IIIB) envelope demonstrated that these CTLs recognize common determinants on diverse HIV strains, including several clinical isolates (*103*). Direct administration of the *env*-expressing vector may provide a more feasible long-term approach and should be accorded a high priority once safety concerns have been addressed. Delivery of naked DNA in the form of circular plasmid DNA engineered to express HIV proteins is also being pursued, with promising results (*104*). This approach offers several advantages, including low cost and the ease of preparation of DNA, and could revolutionize immunization strategies. However, the clinical benefit of any of these approaches in HIV disease remains uncertain.

Certain cytokines, such as tumor necrosis factor (TNF) and interleukin-6 (IL-6), may have a direct up-regulatory effect on HIV synthesis and should be considered as potential targets for intervention (*105*). Reported inhibitors of TNF action, pentoxifylline (Trental) and BRL 61063, are currently being evaluated (*106*). Certain thiol-based agents, such as *N*-acetylcysteine (NAC) and 2-oxothiazolidine-4-carboxylate (OTC, Procysteine), have been reported to prevent activation of HIV in latently infected cells, presumably as a result of the ability to raise the intracellular levels of glutathione, which is required for a variety of immune functions (*107*). More recently, a new class of anti-HIV agents, 1,2-dithiole-3-thiones, exemplified by oltipraz [4-methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione], not only elevated levels of glutathione but also appeared to irreversibly inhibit HIV RT (*108*).

Interferon-α (IFN-α) blocks HIV replication in vitro probably by interfering with the assembly or release (or both) of mature virions (*109*). Clinical trials addressing the in vivo effects of IFN-α, both alone and in combination with other agents, in patients at all stages of HIV-1 infection have suggested that patients at earlier stages may benefit from treatment with this agent (*110*). Controlled clinical trials of IFN-α alone and in combination with antiretrovirals are under way.

$CD8^+$ major histocompatibility complex (MHC) class I–restricted CTLs kill HIV-infected cells in culture and may also block HIV replication by release of a soluble factor (*111*). Expansion and reinfusion of HIV-specific autologous $CD8^+$ T cells from HIV-infected individuals (*112*) is undergoing initial clinical evaluation. Given the difficulty and expense in expanding cells ex vivo, this approach is unlikely to have widespread use in the near future. This early trial, however, may provide valuable information on the role of $CD8^+$ cells in controlling viral replication and may stimulate research on alternative sources of $CD8^+$ cells, including allogeneic or xenogeneic cells or universally accepted CTLs.

Several approaches to broadly reconstitute immune competence are being evaluated. IL-2 induced a transient but significant increase in the number of $CD4^+$ cells (*113*), and low-dose polyethylene glycol–modified IL-2 (PEG–IL-2) increased killer cell activity and enhanced proliferative responses in infected individuals (*114*). Additional trials of PEG–IL-2 in combination with AZT or ddI are under way. Thymic humoral factor (THF) and thymopentin (TP5) are two hormone-based therapies that have entered clinical trial (*115*). THF is reported to augment cell-mediated immunity, whereas thymopentin is reported to enhance T cell function by increasing lymphokine production.

If procedures for purging HIV from infected cells could be developed, it may be worthwhile to pursue ex vivo expansion and reinfusion of $CD4^+$ cells. Finally, a long-range goal to achieve complete immune function would be the administration of multipotent progenitor cells genetically engineered to resist HIV infection. Significant advances in gene transduction and expression in human progenitor cells, and information on the ability of engineered progenitors to differentiate in HIV-infected individuals, will be needed prior to attaining such a lofty goal. In the interim, evaluation of syngeneic bone marrow transplantation and adoptive transfer of peripheral blood lymphocytes in combination with antiretroviral regimens continues (*116*).

## The Future of HIV Therapeutics

Three inhibitors of RT have been licensed, and other inhibitors of RT, Tat, and protease are currently in clinical trial. In addition, several combinations of anti-RT

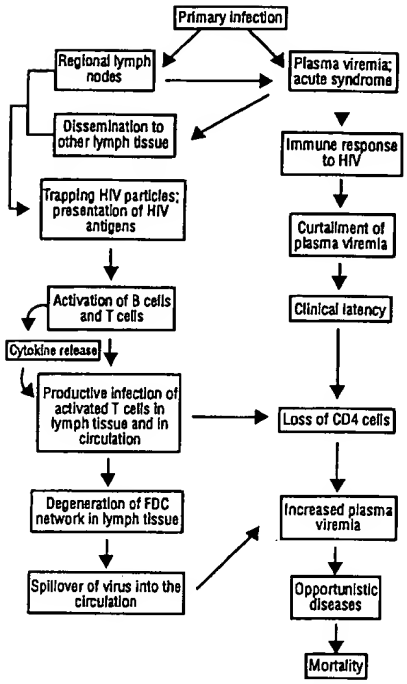


**Fig. 2.** Progression of HIV infection from acute infection to morbidity and mortality. The right side depicts information obtained by monitoring of the blood from infected individuals. The left side summarizes information on the role of lymphoid organs in disease progression. FDC, follicular dendritic cells. [Adapted from (*123*)]

agents are undergoing clinical evaluation, both in simultaneous and alternating regimens. The use of combinations of agents that act at pre- and post-integration events is an attractive theoretical approach. However, there is currently no clinical information proving that mechanistically diverse combinations are more efficacious than single agents or combinations of agents that act at the same step in the viral life cycle. The orderly evaluation of drug combinations offers significant preclinical and clinical challenges. Indeed, it is expected that the use of combinations either to overcome or avoid resistance, to provide a synergistic antiviral effect, or to manage drug-related toxicities will improve the management of HIV disease in the next few years.

It is likely that the speed of discovery of new drugs will be accelerated by newer technologies, such as screens based on combinatorial libraries of peptides and oligonucleotides, permitting the rapid screening of millions of compounds and equally rapid optimization of candidate drugs (*88, 117*).

Although most current antiretroviral approaches are based on an understanding of the life cycle of HIV in cell culture, much less is understood about the interactions of HIV with the host and the mechanisms by which HIV causes disease. As new information from in vivo studies emerges, new therapeutic concepts will likely follow. For example, wild-type SIV containing *nef* and SIV with a *nef* deletion were indistinguishable in their growth kinetics in cultured cells (*118*). Yet, animals infected with SIV deleted in *nef* became infected but did not develop disease, whereas the wild-type virus caused disease and death (*118*).

Information on the earliest stages of disease may also yield valuable clues to new therapeutic strategies. During acute infection, HIV replication may be amplified because a very high percentage of cells are activated, probably by various cytokines that act by autocrine or paracrine routes (Fig. 2) (*105, 118a*). Methods to downregulate this process may prove particularly beneficial. Further, deciphering the specificity of cells or antibodies that clear the early burst of infectious virus from the circulation will facilitate the design of therapeutic immunization strategies. Finally, emerging data suggest that viral burden in early infection is relatively low, and the number of genomic variants appears to be small. Aggressive therapy with antiretrovirals, with or without immune-targeted therapies, might impact long-term outcome. In addition, one of the highest priorities of current research is determining the stage at which antiretroviral therapy should begin, particularly in view of data indicating that viral replication occurs at all stages of disease (*18, 119*).

Recent reports have suggested that the transition from apparent clinical latency to a stage of more rapid decline is associated with a change in the phenotype of the predominant virus in the body, specifically from nonsyncytium-inducing (NSI) to syncytium-inducing (SI) virus (*120*). Whether SI phenotype is more pathogenic or whether it is simply a marker for increased viral replication is not known. Reports have suggested that AZT is only minimally effective against the SI phenotype (*121*). Understanding the process and consequences of the change in phenotype will be very important. In the interim, screening new potential therapies against both NSI and SI types of virus is recommended.

Finally, the processes underlying immune damage, including loss of $CD4^+$ cells and induction of anergy, need to be explored further. Direct killing of cells by HIV may not be the only mechanism of immune damage (*122*), as recently reviewed (*123*). Elucidation of pathogenic mechanisms may not only provide additional targets for intervention but may also guide approaches to augment or restore immune function.

Overall, substantial resources have not been devoted to the pursuit of potential drug targets other than RT and protease, and even fewer resources have been devoted to more innovative and risky approaches to restore immune function. In part, selection of therapeutic approaches has been affected by limitations in basic knowledge and by the existence of technologies suitable for high-throughput screens. Pharmaceutical companies have demonstrated extreme care in managing the overall level of resources devoted to the development of antiviral agents. Most higher risk technologies are supported by venture capital. The success of any particular approach is likely to generate interest from larger companies. In the interim, it is critical that the government continues to support basic research on the pathogenesis of HIV disease and to foster linkages that accomplish a rapid translation of new findings and new technologies into therapeutic gains.

The field of HIV therapeutics would not be where it is today had it not been for previous research on retroviruses that helped identify RT and other viral proteins as targets for therapeutic intervention. The field has progressed to the point where therapies targeted to different stages of replication are in trial and additional mechanism-based targets are in place. It is the view of these authors that ultimate success will depend not only on learning how best to use the drugs that are currently available and in development, but also on improving our understanding of the basic disease process so that all steps of the virus's impact on the host can be identified and countered, if not eliminated.

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# Human Immunodeficiency Virus Type 1 Viral Protein R Localization in Infected Cells and Virions

YUH-LING LU, PAUL SPEARMAN, AND LEE RATNER*

*Departments of Medicine and Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110*

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**The subcellular localization of human immunodeficiency virus type 1 (HIV-1) viral protein R (Vpr) was examined by subcellular fractionation. In HIV-1-infected peripheral blood mononuclear cells, Vpr was found in the nuclear and membrane fractions as well as the conditioned medium. Expression of Vpr without other HIV-1 proteins, in two different eukaryotic expression systems, demonstrated a predominant localization of Vpr in the nuclear matrix and chromatin extract fractions. Deletion of the carboxyl-terminal 19-amino-acid arginine-rich sequence impaired Vpr nuclear localization. Indirect immunofluorescence confirmed the nuclear localization of Vpr and also indicated a perinuclear location. Expression of Vpr alone did not result in export of the protein from the cell, but when coexpressed with the Gag protein, Vpr was exported and found in virus-like particles. A truncated Gag protein, missing the p6 sequence and a portion of the p9 sequence, was incapable of exporting Vpr from the cell. Regulation of Vpr localization may be important in the influence of this protein on virus replication.**

The human immunodeficiency virus type 1 (HIV-1) genome is more complex than those of murine and avian retroviruses. In addition to the basic functions encoded by *gag*, *pol*, and *env*, the HIV-1 genome includes at least six additional genes with distinct regulatory roles (see references 31 and 39 for reviews). Two of these regulatory genes, *tat* and *rev*, are essential for virus gene expression. The remaining genes, *vpr*, *vpu*, *vif*, and *nef* are dispensable for virus replication in tissue culture, but mutations of these genes alter the replication properties of the virus.

HIV-1 *vpr* encodes a protein (viral protein R [Vpr]) of 96 amino acids (27). Previous studies have shown that the *vpr* products can increase the rate of replication of the virus and accelerate its cytopathic effects in T-cell lines and in peripheral blood mononuclear cells (PBMCs) (6, 8, 28, 29). Cohen and colleagues suggested that *vpr* increased gene expression from the HIV-1 promoter, as well as a wide range of other promoters, but the mechanism of this effect remains to be determined (7). *vpr* is also found in the genomes of HIV-2 and several strains of simian immunodeficiency virus (SIV) (5, 14). The activity of the HIV-2 and SIV *vpr* gene products appears to be similar to that of HIV-1 *vpr* (16, 35). Furthermore, $SIV_{mac}$ *vpr* is important for the development of an AIDS-like disease in rhesus macaques (22).

HIV-1, HIV-2, and SIV *vpr* gene products have 26 to 36% amino acid identity (40). Certain features of the Vpr proteins among different HIV isolates are highly conserved, including the presence of a single cysteine residue at amino acid position 76 of HIV-1 Vpr, a predicted amphipathic alpha-helical loop in the N-terminal portion of the protein, and the presence of an arginine-rich carboxyl-terminal tail.

Vpr is packaged within the HIV-1 virion (6, 45). Similar findings have been reported for $SIV_{mac}$ Vpr (44). The Vpr protein is the only regulatory product of HIV-1 found in virus particles, though the homologous *vpx* gene products of HIV-2 and $SIV_{mac}$ are also associated with virus particles (17–19). However, the subcellular distribution of Vpr and the mechanism of incorporation into virus particles are unclear. In this study, the subcellular localization of Vpr in HIV-1-infected PBMCs and in two different *vpr* expression systems in mammalian cells was examined by subcellular fractionation and indirect immunofluorescence techniques. The role in cellular localization of the carboxyl-terminal arginine-rich sequence of Vpr was specifically studied. Lastly, the effects of Gag coexpression on Vpr export and incorporation into virus particles were examined.

## MATERIALS AND METHODS

**Cell lines and culture.** COS-7 cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 1 mM pyruvate, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. BSC40 cells were maintained in the same medium. PBMCs were purified from normal human leukocytes by centrifugation onto Ficoll. After 3 days of stimulation with phytohemagglutinin (15 μg/ml; Sigma), PBMCs were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 4 mM glutamine, 50 U of recombinant interleukin 2 (Cetus) per ml, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

**Vpr and Gag expression plasmids.** HIV-1 nucleotides 5558 to 5869 (numbered according to reference 27) encompassing the pNL4-3 *vpr* gene was obtained by the polymerase chain amplification reaction, using primers AATACCATGGAA CAAGCCCCAGAAGA and GATGCTTCCAGGGATCCGT CTAGGATCTACTG. The reaction product was digested with *Nco*I and *Bam*HI and cloned into pTM3 (designated here pTM) (12, 26), to produce pTM-VPR. The *Nco*I-*Bam*HI fragment of pTM-VPR was cloned between the *Sal*I and *Sac*I sites of pSRalpha (25) after blunt ending with T4 DNA polymerase, in the correct orientation (pSR-VPRs) and in the incorrect or antisense orientation (pSR-VPRa) (see Fig. 2A). The CRST mutant clone was constructed by digestion of pNL4-3 with SalI, at nucleotide 5786, and blunt ending with the Klenow fragment of DNA polymerase I. It was then cloned

* Corresponding author.

into the pTM vector by the strategy used to construct pTM-VPR.

An *Nco*I-*Nco*I fragment from plasmid pGG1 (3, 32), containing nucleotides 789 to 5674, was cloned into the *Nco*I site of plasmid pTM3, to produce plasmid pTM-GAG-POL. This clone contains the *gag* and *pol* open reading frames. Expression of the *pol* gene was abrogated by frameshift mutation at the *Bcl*I site at nucleotide 2428 in the 5′ portion of *pol* to produce pTM-GAG. Plasmid pTM-GAG(p41) was constructed from pTM-GAG-POL by frameshift mutation at the *Apa*I site at nucleotide 2005, using T4 DNA polymerase I. This results in a termination codon at nucleotide 2058 after the first Cys-His box coding region of p9.

**Vpr and Gag p24 antisera.** A New Zealand White rabbit was inoculated with complete Freund's adjuvant containing 200 μg of Vpr protein, synthesized according to the sequence of HIV-1 strain LAI and kindly provided by H. Gras-Masse (13). Booster doses of 200 μg of Vpr in incomplete Freund's adjuvant were given at 3, 6, 9, and 18 weeks after the initial inoculation. A New Zealand White rabbit was inoculated with complete Freund's adjuvant containing 100 μg of recombinant p24 protein (provided by American Biotechnology through the NIH AIDS Research and Reference Reagent Program), and 100-μg booster doses were given 2 and 4 weeks later.

**Virus infection.** HIV-1 virus stocks were generated by transfection of 60% confluent 10-cm-diameter COS-7 plates with 10 μg of recombinant proviral clone NLHXADA(GG) (41) and 2 μg of pCV1 (*tat* expression vector [1]) by the calcium phosphate precipitation method, followed 5 h later by 10% dimethyl sulfoxide shock for 2 min. The cells were washed twice with phosphate-buffered saline (PBS) before refeeding with 10 ml of fresh medium. Culture supernatants were harvested after 48 h and filtered (0.2-μm-pore-size Millipore filter). Five milliliters of culture supernatant was used to infect $5 \times 10^7$ PBMCs. Virus replication was monitored by determination of reverse transcriptase activity (30). PBMCs ($10^7$) were labeled for 20 h in 2 ml of leucine-free RPMI 1640 medium containing 200 μCi of [4,5-$^3$H]leucine and fractionated as described below. Mock-infected cultures were exposed to 5 ml of filtered culture supernatants from untransfected COS-7 cells.

**Transfection and radiolabeling of COS-7 cells.** COS-7 cells were grown to 60% confluence on 10-cm-diameter culture dishes and transfected with 15 μg of pSR-VPRs or pSR-VPRa by lipofection as recommended by GIBCO. Briefly, 15 μl of Lipofectin (GIBCO) was mixed with 3 ml of Opti-MEM I reduced-serum medium (GIBCO), and then 15 μg of DNA was added. The mixture was allowed to incubate at room temperature for 10 min before addition of the cells. Forty-eight hours after transfection, the cells were labeled with 4 ml of leucine-free DMEM containing 100 μCi of [4,5-$^3$H]leucine per ml for 40 h.

**Infection-transfection protocol for the vaccinia virus expression system.** BSC40 cells were grown to 90% confluence on 10-cm-diameter plates, infected for 1 h at 37°C with vTF7-3 (12, 26) at a multiplicity of infection of 10, and transfected with pTM vectors by the lipofectin transfection method. Four hours after transfection, the cells were labeled for 20 h with 3 ml of leucine-free DMEM containing 100 μCi of [4,5-$^3$H]leucine per ml.

**Subcellular fractionation.** Labeled cells were fractionated into membrane, cytosolic, postnuclear, and nuclear fractions as previously described (23), with minor modifications. Nuclei were further fractionated into nucleoplasm, chromatin extract, and nuclear matrix as described by Staufenbiel and Deppert (37). Cells were washed with ice-cold PBS and scraped in PBS. The cell pellet volume was measured and resuspended in 10 volumes of Dounce buffer (10 mM Tris-HCl [pH 7.5], 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM dithiothreitol). The cells were allowed to swell on ice for 10 min before disruption with 20 to 25 strokes of a Dounce homogenizer. A small aliquot was saved and mixed with an equal volume of 0.4% (wt/vol) trypan blue in PBS to examine cell disruption under phase microscopy. Dounce homogenization was continued until >99% cells were disrupted. The homogenate was centrifuged at 1,500 rpm for 10 min in a Beckman GS-6 rotor to generate the supernatant containing both the membrane and cytosolic fractions and the nuclear pellet.

The nuclear pellet was subsequently extracted by four steps. First, the nuclear pellet was resuspended in buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES-KOH; pH 7.4], 0.25 M sucrose, 0.2 mM PMSF, 0.5 mM dithiothreitol) supplemented with 0.1% (vol/vol) Triton X-100 and then incubated for 15 min on ice. The nuclei were pelleted at 1,500 rpm for 10 min, and the supernatant was designated the postnuclear wash fraction. Second, the pellet was resuspended in buffer A supplemented with 0.5% Nonidet P-40 and incubated for 30 min on ice. The nuclei were pelleted again at 1,500 rpm for 10 min, and the supernatant was designated the nucleoplasmic fraction. The latter procedure was repeated twice, and the supernatants were pooled. Third, the Nonidet P-40-extracted nuclear pellet was subjected to DNase I digestion (1% [vol/vol] Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM PMSF, and 50 μg of DNase I [Sigma] per ml in PBS) for 15 min at 37°C. Then an equal volume of 4 M NaCl was added, and incubation was continued for 30 min at 4°C. The sample was then subjected to centrifugation at 2,500 rpm for 10 min. The supernatant was designated the chromatin extract, and the pellet was resuspended in radioimmunoprecipitation assay (RIPA) buffer (1% [vol/vol] Triton X-100, 0.5% [wt/vol] deoxycholate, 0.1% [wt/vol] sodium dodecyl sulfate [SDS], and 0.2 mM PMSF in PBS) for 30 min on ice. The insoluble portion was removed by centrifugation at 1,000 rpm for 15 min. The supernatant was designated the nuclear matrix. The purity of the nuclei was examined by using a control cytosolic protein, β-galactosidase, expressed in the same cells by transfection of a cDNA expression clone. More than 98% of the β-galactosidase activity was found in the cytosol, as measured by enzymatic assay. Only 1.4% of the β-galactosidase activity was detected in the postnuclear wash fraction. No detectable activity was found in the purified nuclei.

For the membrane and cytosolic fractions, the salt concentration was adjusted to 0.15 M NaCl and then the preparations were fractionated by ultracentrifugation at 100,000 × *g* for 30 min. The supernatant was designated the cytosolic fraction. The pellet was washed with 1 M NaCl in PBS for 30 min on ice, and ultracentrifugation was repeated. The supernatant was designated the membrane wash fraction, and the membrane pellet was resuspended in RIPA buffer.

**Immunoprecipitation.** Equivalent proportions (volume/volume) of each of the subcellular fractions were precipitated overnight with 10% trichloroacetic acid at 4°C. The resulting pellets were washed in 70% ethanol, solubilized in sample buffer (0.125 M Tris-HCl [pH 6.8], 20% glycerol, 10% [vol/vol] 2-mercaptoethanol, 4% [wt/vol] SDS). An aliquot was taken for scintillation counting to determine relative labeled protein content.

Equivalent proportions (volume/volume) of each of the subcellular fractions were adjusted to 500 μl of RIPA buffer. Immunoprecipitation was performed by the addition to 500 μl of each fraction 5 μl of anti-Vpr antiserum or 5 μl of anti-Gag

antiserum. Incubation was continued overnight at 4°C. Twenty microliters of protein A-Sepharose beads (50% [vol/vol] in PBS) was added, and the mixture was incubated for 120 min at 4°C. Immunoprecipitates were collected at 500 × *g* for 3 min at room temperature and washed three times with RIPA buffer. The beads were resuspended in 30 μl of sample buffer. Samples were treated at 100°C for 10 min before SDS–12% polyacrylamide gel electrophoresis (PAGE), fixation for 30 min in 25% isopropanol–10% acetic acid, treatment with Amplify (Amersham), and autoradiography were performed. Band intensities were determined by densitometry.

**Immunofluorescence.** BSC40 cells ($10^4$) were plated on eight-well Lab-Tek chamber slides overnight. The cells were infected with vTF7-3 and transfected as described above. The cells were fixed with 2.5% (wt/vol) glutaraldehyde for 15 min and permeabilized with 0.2% (vol/vol) Triton X-100 for 6 min at room temperature. The cells were then blocked for nonspecific binding of immunoglobulin by incubation for 30 min with PBS containing 5% (wt/vol) nonfat dry milk and 0.1% (vol/vol) Tween 20. Slides were then incubated with rabbit anti-Vpr antibody (1:100 in Tween buffer [PBS with 0.5% Tween 20 and 1% bovine serum albumin]) and mouse monoclonal antihistone antibody (1:500 in Tween buffer; Chemicon) for 1 h at room temperature. The cells were washed several times with 0.3% (vol/vol) Triton X-100 in PBS and incubated at 4°C for 60 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G to detect Vpr and rhodamine-conjugated goat anti-mouse immunoglobulin G to detect histones. The slides were washed extensively with PBS and mounted in Aqua mount solution (Lerner Lab) containing 2.5% 1,4-diazobicyclo-[2,2,2]-octane (Sigma) to prevent photobleaching of the FITC signal. Slide preparations were examined on a Nikon fluorescence microscope equipped with appropriate filters and a microflex UFX camera system. Photographs were prepared by using Kodak T-MAX film, push processed to ASA 3200.

**Sucrose gradients.** BSC40 cells were infected, transfected, and labeled with [$^3$H]leucine as described above. Cellular debris was removed from the conditioned medium by centrifugation at 2,500 rpm for 15 min in a Beckman GS-6 rotor. Particles were concentrated by sedimentation through a 20% sucrose cushion prepared in PBS at 28,000 rpm for 90 min at 4°C in an SW28.1 rotor. Particles were resuspended in 200 μl of PBS, layered on a linear 20 to 60% sucrose gradient in PBS, and centrifuged in an SW28.1 rotor at 20,000 rpm for 16 h at 4°C. Fractions were collected from the top of the tube.

## RESULTS

**Localization of Vpr in HIV-1-infected PBMCs.** To examine the intracellular localization of Vpr, PBMCs, a natural target cell population, were chosen for HIV-1 infection. HIV-1 strain NLHXADA(GG) was chosen since it encodes a functional 96-amino-acid form of Vpr identical in amino acid sequence to that encoded by NL4-3 (27, 29, 42). Nine days after infection, the cells were labeled for 20 h with [$^3$H]leucine, lysed by Dounce homogenization, and then fractionated into nuclear, cytosolic, and membrane fractions by differential centrifugation. Each fraction was immunoprecipitated with a polyclonal rabbit anti-Vpr antiserum and subjected to SDS-PAGE (Fig. 1). A Vpr-specific protein of 14 kDa was detected in NLHX ADA(GG)-infected cells and conditioned medium but not in mock-infected cultures. This protein was not immunoprecipitated with a control antiserum obtained from the prebleed serum of the same rabbit prior to inoculation with the synthetic

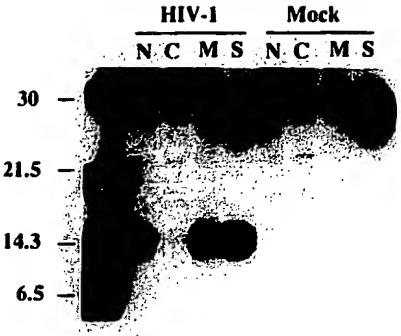


FIG. 1. Localization in PBMCs of Vpr expressed from infectious virus. PBMCs were infected for 9 days with HIV-1 strain NLHX ADA(GG) or were mock infected. The cells were labeled with [$^3$H]leucine, the medium was harvested (S), and the cells were fractionated into nuclear (N), cytosolic (C), and membrane (M) fractions as described in Materials and Methods. Equivalent portions of each fraction were immunoprecipitated with the anti-Vpr antiserum and analyzed by SDS-PAGE. Molecular mass markers are shown at the left in kilodaltons.

Vpr used for production of the anti-Vpr antiserum (not shown).

The majority of Vpr was found in the culture supernatant, consistent with previous reports that Vpr is virion associated (6, 45). Intracellular Vpr was found in both nuclear (26%) and membrane (20%) fractions. Vpr in the nuclear fraction was not due to contamination with unbroken cells, which made up <1% of the total cell population used in the fractionation experiments. Less than 4% of the Vpr was found in the cytosolic fraction. Approximately 50% of the labeled Vpr was found in the conditioned medium.

**Expression and localization of Vpr in mammalian cells.** To study the cellular localization of Vpr without the effect of other HIV-1 components, the NLHXADA(GG) *vpr* gene was cloned into a simian virus 40 (SV40)-based vector system, pSRalpha, in both the correct (pSR-VPRs) and incorrect or antisense (pSR-VPRa) orientations (Fig. 2A). The expression plasmid contains both the SV40 early enhancer (SV40-ori) and a human T-cell leukemia virus type I (HTLV-I) promoter with R and U5 elements of the HTLV-I long terminal repeat. This expression plasmid has previously been reported to achieve high levels of expression of a number of different lymphokine cDNAs in a variety of cell types (38) and to facilitate the expression of HIV-2 *vpx* in COS-1 cells (21).

pSR-VPRs and pSR-VPRa were transfected into COS-7 cells, and the cells were labeled with [$^3$H]leucine and separated into membrane, membrane wash, cytosolic, and nuclear fractions. Membranes loosely associated with nuclei were removed by a wash with 0.1% Triton X-100 and were designated the postnuclear wash. Soluble nucleoplasmic proteins were extracted with two successive washes in 0.5% Nonidet P-40, which permeabilizes the nuclear membrane (2). This method has previously been demonstrated to preserve overall nuclear and nucleolar architecture (33). The chromatin fraction was obtained by digestion of the resultant insoluble nuclear fraction with DNase I and by a subsequent wash in a high-salt buffer. This fraction contained all of the major histone proteins found in intact nuclei (not shown). The salt- and detergent-insoluble fraction was pelleted to yield the nuclear matrix fraction, which was solubilized in RIPA buffer.

The partition of Vpr during fractionation was examined by immunoprecipitation with the anti-Vpr antibody, SDS-PAGE, and densitometric quantitation (Fig. 2B). The chromatin frac-

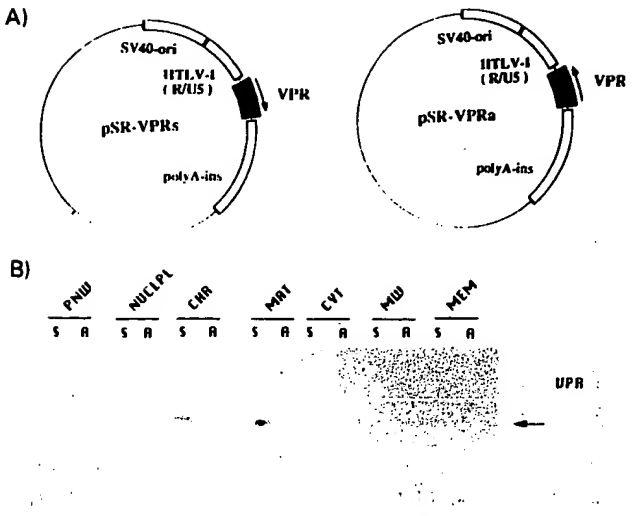


FIG. 2. Localization in COS-7 cells of Vpr expressed from pSR. (A) Vpr sense (pSR-VPRs) and antisense (pSR-VPRa) expression plasmids, which include a transcriptional enhancer (SV40-ori), a transcriptional promoter (HTLV-I long terminal repeat [R/U5]), and a polyadenylation insertion sequence (polyA-ins). (B) Subcellular fractionation of Vpr expressed in transfected and [$^3$H]leucine-labeled COS-7 cells from pSV-VPRs (S) and pSR-VPRa (A) in postnuclear wash (PNW), nucleoplasm (NUCLPL), chromatin (CHR), nuclear matrix (MAT), cytosol (CYT), membrane wash (MW), and membranes (MEM). Equivalent amounts of each fraction were immunoprecipitated with the anti-Vpr antiserum and analyzed by SDS-PAGE. The electrophoretic position of Vpr is shown by an arrow at the right.

tion included 17% of the intracellular labeled proteins and 44% of the total Vpr. The nuclear matrix included 1% of the intracellular labeled proteins and 56% of the total Vpr. Less than 1% of the total Vpr was found in the other cellular fractions.

**Truncation of the C terminus of Vpr impairs nuclear localization.** Most nuclear localization signals consist of a short stretch of positively charged amino acids (15). Interestingly, the C terminus of Vpr contains a high proportion of positively charged amino acids, including 7 arginine residues among the C-terminal 20 amino acids (Fig. 3A). To characterize the role of this C-terminal sequence, a vaccinia virus expression system was used to achieve high-level and rapid expression of Vpr. The NLHXADA(GG) *vpr* gene was cloned into pTM3, a plasmid utilizing a T7 promoter for heterologous gene expression. This plasmid was designated pTM-VPR. A carboxyl-terminal truncation mutant of pTM-VPR, pTM-CRST, was constructed by frameshift mutation at the *SalI* site. A recombinant vaccinia virus, vTF7-3, which encodes T7 RNA polymerase was used for expression in mammalian cells.

BSC40 cells were infected with vTF7-3 and transfected with pTM-VPR or pTM-CRST. Cells were labeled with [$^3$H]leucine, cell supernatants were harvested, and disrupted cells were fractionated into membrane, membrane wash, cytosolic, and postnuclear wash fractions and various nuclear fractions (nucleoplasmic proteins, chromatin, and nuclear matrix). Equivalent amounts of each fraction were immunoprecipitated with the anti-Vpr antiserum and analyzed by SDS-PAGE (Fig. 3B), and band intensities were quantitated by laser densitometry (Fig. 3C). The predominant Vpr product expressed from pTM-VPR had an electrophoretic mobility of a 14-kDa protein, with a minor band with a mobility of a 13-kDa protein. Vpr expressed from pTM-CRST electrophoresed as a 12-kDa protein, consistent with the removal of 17 amino acids from the carboxyl terminus.

Eighty-four percent of pTM-VPR-expressed Vpr was found in nuclear fractions, primarily the nuclear matrix and chromatin fractions (Fig. 3B [left] and C). This result is in agreement with the fractionation data with pSR-VPR-expressed Vpr (Fig. 2). Eight percent of pTM-VPR-expressed Vpr was tightly associated with the membrane fraction (Fig. 3B and C, MEM). The possible discrepancy in the amount of membrane association of Vpr expressed with the vaccinia virus expression system compared with the data obtained with pSR-VPR (Fig. 2) may be related to the significantly higher level of expression of Vpr with the vaccinia virus expression system than with the SV40 plasmid expression system. Only 4% of Vpr was found in the cytosol (Fig. 3B and C, CYT), and no detectable Vpr was released from cells into the cell supernatant.

Deletion of the arginine-rich C terminus of Vpr resulted in a dramatic shift of Vpr cellular localization (Fig. 3B [right] and C). Only 25% of the truncated Vpr was retained in the nuclear fraction. Furthermore, the distribution in nuclear fractions of pTM-CRST product was distinctly different from that of pTM-VPR, with the majority of the truncated protein in the nucleoplasm. Twenty-four percent of the pTM-CRST protein was in the postnuclear wash, compared with 3% of the pTM-VPR product. Thirty-eight percent of the mutant Vpr was found in the cytosol, compared with 4% of the parental Vpr. Similar amounts of pTM-CRST and pTM-VPR products were bound to membranes.

**Indirect immunofluorescence localization of Vpr.** Subcellular fractionation experiments indicated predominant localization of Vpr in the nucleus. To confirm these results, indirect immunofluorescence was performed with fixed cells. BSC40 cells were infected with vTF7-3 and then transfected with pTM-VPR or pTM. Vpr was detected by anti-Vpr rabbit antibody and visualized with FITC-conjugated anti-rabbit antibody. Intense immunofluorescence was observed in the majority of cells transfected with pTM-VPR (Fig. 4A, left), but no fluorescence was observed in cells transfected with the vector pTM alone (Fig. 4A, right) or if preimmune serum was used (not shown).

Four types of staining patterns were observed in four independent experiments in which 50 cells were randomly selected and enumerated. Sixty-two percent of the cells showed a diffuse nuclear and focal perinuclear staining pattern (Fig. 4B, middle). The nucleus is visualized by phase-contrast microscopy (Fig. 4B, left) and mouse antihistone and rhodamine-conjugated anti-mouse antibody (Fig. 4B, right). Twenty-seven percent of the cells showed focal perinuclear staining only. Six percent of the cells had diffuse perinuclear staining with intense immunofluorescence surrounding the nucleus. Four percent of the cells showed only diffuse nuclear staining.

**Influence of Gag protein on Vpr export and virion incorporation.** In HIV-1-infected cells, Vpr was found to be exported into the medium in virus particles (Fig. 1) (6, 45), though no export was found when Vpr was expressed in the absence of other virion components (Fig. 3). To assess the requirements for export, Vpr was coexpressed with the HIV-1 Gag p55 precursor protein by using plasmid pTM-GAG. BSC40 cells were infected with vTF7-3 and transfected with pTM-VPR alone, pTM-GAG alone, or both plasmids. No differences were noted in the electrophoretic mobility or quantity of the 14-kDa product in the cell lysates with pTM-VPR expressed in the presence or absence of pTM-GAG (Fig. 5A, left). The pTM-GAG product was primarily a 55-kDa protein, with smaller amounts of 43- and 41-kDa products. The latter proteins were found to be Gag proteins, since they did not react with a

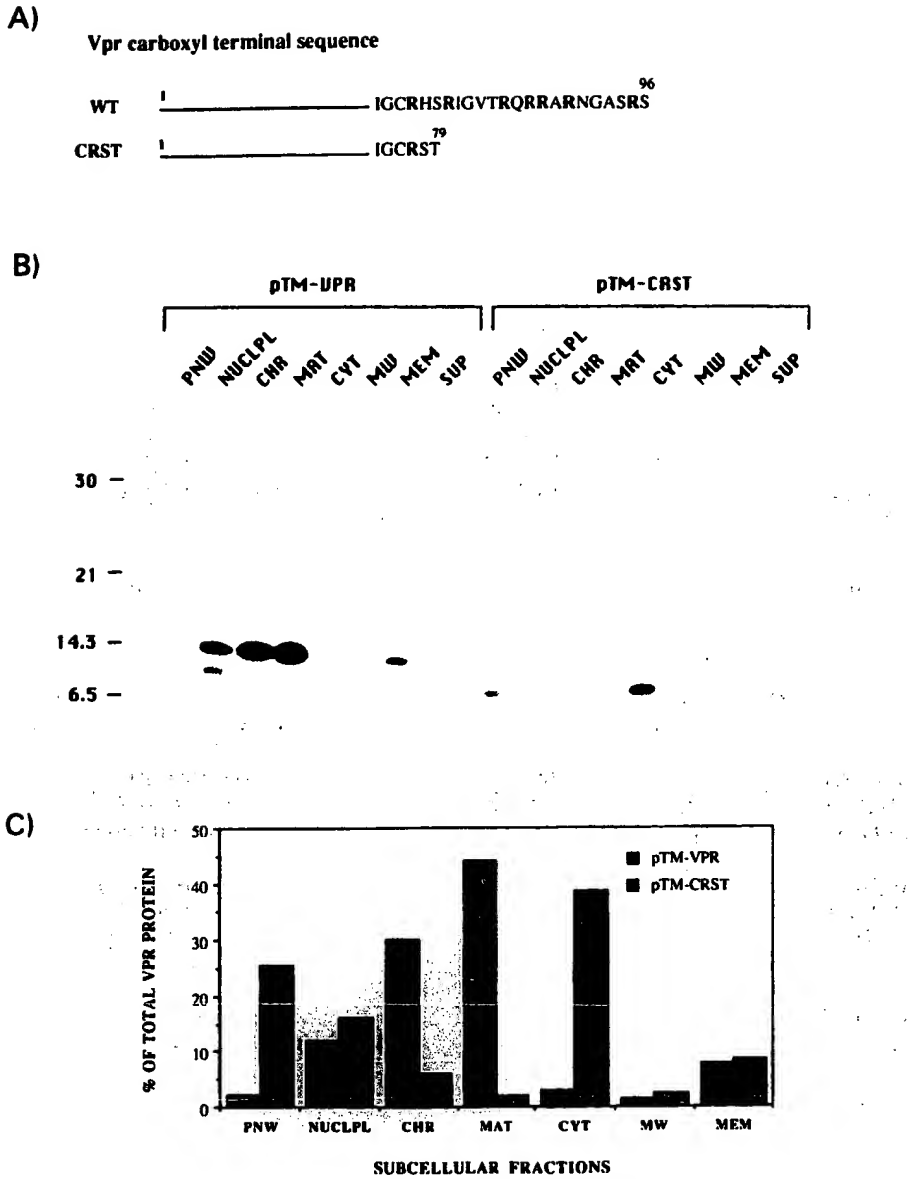


FIG. 3. Subcellular distribution of vaccinia virus-expressed parental Vpr and carboxyl-terminal truncation mutant CRST in BSC40 cells. (A) Schematic drawing of the Vpr protein, indicating the carboxyl-terminal arginine-rich sequence of the wild type (WT) and of the truncation mutant, CRST. (B) Vpr expressed from pTM3 in vTF7-3-infected cells. Cells were labeled with [$^3$H]leucine and fractionated into postnuclear wash (PNW), nucleoplasm (NUCLPL), chromatin (CHR), nuclear matrix (MAT), cytosol (CYT), membrane wash (MW), membranes (MEM), and cellular supernatant (SUP). Molecular mass markers are shown at the left in kilodaltons. (C) Proportion of VPR in each subcellular fraction as determined by laser densitometry from pTM-VPR (solid bars)- or pTM-CRST (hatched bars)-transfected cells.

preimmune serum or the anti-Vpr antibody (not shown). These smaller proteins may represent nonspecific cleavage products, products from initiation at a downstream AUG codon, or premature translational termination. No effects on Gag protein expression were noted with coexpression of Vpr.

Expression of pTM-VPR alone did not result in export in the cell supernatant (Fig. 5A, right). Expression of pTM-GAG resulted in the 55-kDa product in the cell supernatant. Coexpression of pTM-GAG with pTM-VPR promoted the export of Vpr into the cell supernatant.

To determine whether the viral proteins released into the cell supernatant were associated with particles, sucrose gradient analysis was performed (Fig. 6). Particles were first concentrated from the cell supernatant samples by centrifugation through a 20% sucrose cushion. The resultant particulate material was resuspended and analyzed on a linear 20 to 60% sucrose gradient. Each fraction was concentrated with 10% trichloroacetic acid and analyzed by SDS-PAGE. No particle-associated protein was found from cells transfected with pTM-VPR alone (Fig. 6A). Expression of pTM-GAG alone resulted in particle-associated Gag protein banding in fractions 10 and 11, at a density of 1.16 to 1.17 g/ml (Fig. 6B). Expression of pTM-GAG together with pTM-VPR resulted in cosedimentation of both Vpr and Gag in fractions 11 and 12, at a density of 1.16 to 1.17 g/ml (Fig. 6C).

Vpr packaging was also assessed with a clone expressing a

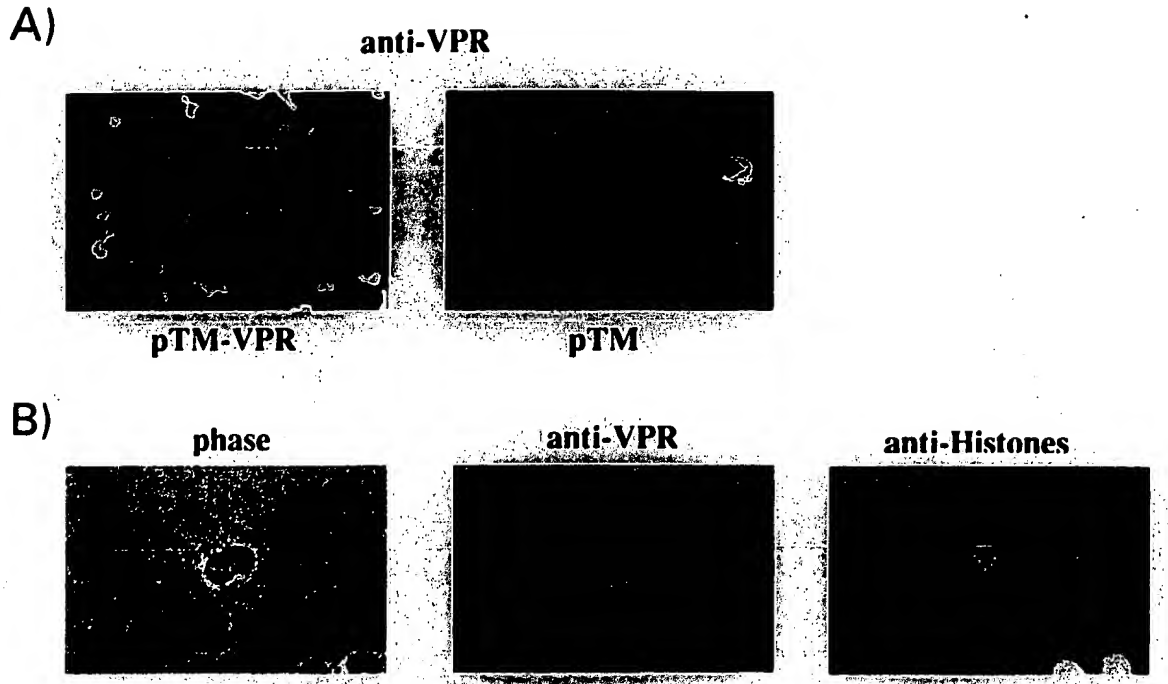


FIG. 4. Immunofluorescence localization of Vpr in BSC40 cells. (A) pTM-VPR (left)- or pTM (right)-transfected vTF7-3 infected cells were incubated with the anti-Vpr antiserum and an FITC-conjugated goat anti-rabbit immunoglobulin. Magnification, ×188. (B) Higher magnification (×752) by phase-contrast microscopy (left) and fluorescence microscopy (middle and right) of a representative cell incubated with anti-Vpr antiserum and FITC-conjugated goat anti-rabbit immunoglobulin (middle; filter with excitation range of 450 to 490 nm and emission range of 520 to 560 nm) and antihistone antiserum and rhodamine-conjugated goat anti-mouse immunoglobulin (right; filter with excitation range of 510 to 560 nm and emission range of >590 nm). Diffuse nuclear (thin arrow) and focal perinuclear staining (thick arrow) are indicated in the middle panel.

truncated form of the Gag precursor protein, pTM-GAG(p41), in which all of the amino acids following the first Cys-His box of NC as well as the C-terminal p6 coding sequence were removed. This construct has been shown to produce virus-like particles in the vaccinia virus infection-transfection system in a manner similar to that of pTM-GAG (not shown). When coexpressed with pTM-VPR, p41 appeared in the supernatant but did not result in the export of Vpr from the cells (Fig. 5B). The failure to detect Vpr in the cell supernatant was due to the absence of Vpr export rather than to the lower quantity of p41 Gag particles produced, since no Vpr was detected, even after overexposure of the autoradiogram shown in Fig. 5B. In contrast, in the same experiment, production of p55 from pTM-GAG resulted in significant export of Vpr.

## DISCUSSION

**Localization of Vpr in the nucleus.** In this study, we used three different expression systems to provide evidence for the localization of a significant proportion of Vpr in the nucleus, as demonstrated by subcellular fractionation techniques. In HIV-1-infected PBMCs, 26% of the expressed Vpr was found in the nucleus (Fig. 1). In contrast, when Vpr was expressed in the absence of other viral components by using an SV40 expression plasmid, almost all of the protein was found in the nucleus (Fig. 2). Similar results were obtained with the vaccinia virus expression system, in which case 84% of the Vpr was found in the nucleus (Fig. 3). Results of the indirect immunofluorescence experiments support the results obtained by using subcellular fractionation techniques, indicating nuclear staining in 66% of Vpr-expressing cells (Fig. 4; see Results).

Further fractionation of the isolated nuclei provides additional evidence for Vpr localization in the nucleus rather than in membranes loosely associated with the nuclear membranes. These experiments identified the predominant association of Vpr with the chromatin and nuclear matrix fractions (Fig. 2 and 3). The association of Vpr with the nuclear matrix is unlikely to be spurious, since it is resistant to Nonidet P-40, DNase, and high-salt extraction procedures. Although the role of the nuclear matrix in transcriptional regulation is unclear, several studies have indicated that it may play an important role. The nuclear matrix has been reported to have a role in mRNA transcription and processing via its involvement in attachment and/or association with newly transcribed mRNA (20), ribonucleoprotein particles (11), and pre-mRNA splicing machinery (36, 46). Several gene products, characterized for their ability to promote oncogenic transformation, are also associated with the nuclear matrix. These include the large T antigen of polyomavirus (4), *myc* gene products (9), the adenovirus E1A protein (10), and the Tax protein of HTLV-I (43). The presence of Vpr in the nuclear matrix might indicate a role in *trans* activation of viral gene expression or RNA processing. This is consistent with a report by Cohen and colleagues suggested that Vpr may serve as a *trans* activator of HIV-1 gene expression as well as a *trans* activator of other genes (7). However, the mechanism of this effect and its relevance to Vpr action during virus replication remain unclear. Alternatively, Vpr association with the nuclear matrix may affect host cell gene expression. This view is consistent with a recent report that Vpr induces muscle cell differentiation (24).

Though Vpr lacks a classical nuclear localization signal (15), the carboxyl-terminal portion of the protein is rich in basic amino acids. A truncation mutation which removes the carboxyl-terminal 19 amino acids was found to impair Vpr localiza-

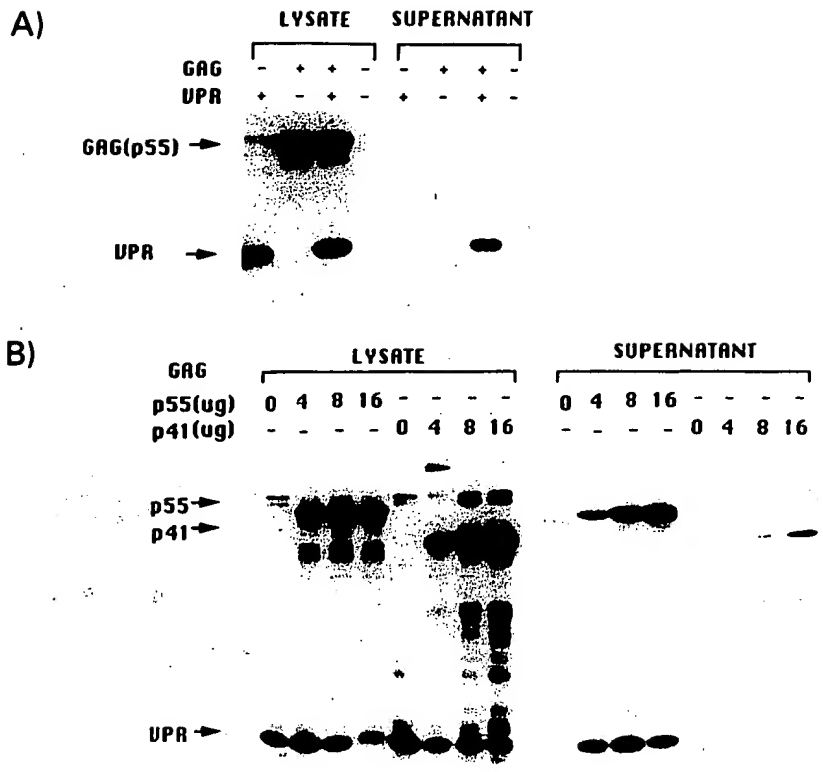


FIG. 5. Influence of Gag expression on Vpr localization. (A) BSC40 cells ($10^6$) were grown on 60-mm-diameter plates overnight and then infected with vTF7-3 and transfected with 7.5 μg of pTM-VPR (VPR) and/or 7.5 μg of pTM-GAG (GAG). The negative control cells were transfected with 15 μg of pTM vector. The cells were labeled with 100 μCi of [$^3$H]leucine per ml for 16 h, scraped in PBS, and resuspended in RIPA buffer. The cell lysates and cell supernatant fractions were immunoprecipitated with both the anti-Vpr and anti-Gag antisera and analyzed by SDS-PAGE. The electrophoretic positions of Gag and Vpr are shown at the left. (B) BSC40 cells ($0.3 \times 10^6$) on 35-mm-diameter plates were infected and transfected with 2 μg of pTM-VPR and 0, 4, 8, or 16 μg of pTM-GAG(p55) or pTM-GAG(p41), as indicated at the top. The cells were labeled and analyzed as described above.

tion in the nucleus (Fig. 3). Furthermore, the distribution of the small proportion of truncated Vpr found in the nucleus was distinctly different from that of full-length Vpr, with the truncated Vpr localized predominantly in the nucleoplasm and very little Vpr in the nuclear matrix or chromatin fractions. It is possible that truncation of the carboxyl-terminal portion of Vpr alters the conformation of the molecule. Alternatively, it is possible that the carboxyl-terminal arginine-rich sequence serves as at least part of a nuclear localization signal. This view is supported by our preliminary observations that attachment of the C-terminal 19-amino-acid Vpr sequence onto β-galactosidase directs this protein to the nucleus (not shown).

Previous studies with lymphoid cells had indicated an important functional role for the C-terminal Vpr sequence (29). Therefore, these findings are consistent with an important role for Vpr localization in the nucleus for HIV-1 replication.

**Membrane-associated Vpr.** Though very little Vpr could be identified in the cytosol, a small proportion was consistently associated with the membrane fraction. This observation is in agreement with findings of Sato and colleagues (34). In HIV-1-infected PBMCs, 20% of Vpr was found in the membrane fraction (Fig. 1), whereas with the vaccinia virus expression form of the protein, 8% was found in the membrane (Fig. 3B and C). The indirect immunofluorescence experiments also suggested that some Vpr is found at an extranuclear site but closely associated with the nucleus (Fig. 4; see Results). The latter site may represent intracellular membranes, possibly with either the endoplasmic reticulum or Golgi apparatus. However, a Golgi location for Vpr is unlikely, since brefeldin A treatment did not change Vpr localization (not shown). The nature and significance of membrane localization of VPR require further analysis.

**VPR export from cells and incorporation into virus particles.** Several previous studies have demonstrated that HIV- and SIV-expressed Vpr is incorporated into virus particles (6, 44, 45). This finding is in agreement with our observation that 50% of Vpr expressed in HIV-1-infected PBMCs is exported from the cells (Fig. 1). Vpr expression in the absence of other viral components resulted in no detectable export (Fig. 3 and 5). However, coexpression with the Gag p55 precursor protein resulted in export of VPR from the transfected cells (Fig. 5) and incorporation into virus-like particles (Fig. 6). Thus, Vpr incorporation into virus particles is independent of viral envelope incorporation. This finding suggests that Vpr associates directly or indirectly with a portion of the Gag precursor protein. The finding that the p41 truncation form of Gag is unable to package Vpr suggests the possibility of an interaction between either the p9 nucleocapsid protein or the proline-rich p6 protein and Vpr.

Although the significance of Vpr incorporation into virions

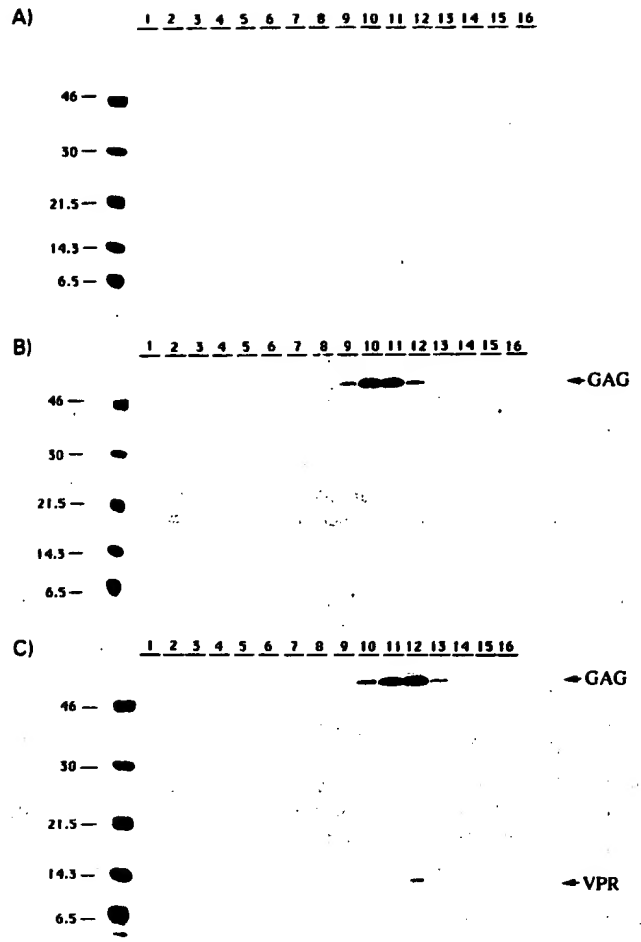


FIG. 6. Sucrose gradient analysis of particles released from BSC40 cells transfected with pTM-VPR (A), pTM-GAG (B), pTM-VPR and pTM-GAG (C). Fractions were precipitated with 10% (wt/vol) trichloroacetic acid and analyzed by SDS-PAGE. Fraction 1 is from the bottom and fraction 16 from the top of each gradient. Molecular mass markers are shown at the left in kilodaltons.

is unclear, it is likely that this protein plays an important role in early events in the virus life cycle. It is tempting to speculate that the nuclear localization domain of Vpr allows targeting of the viral preintegration complex to the nucleus. Further studies on this important regulatory protein will be required to fully elucidate its role in the HIV life cycle.

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# A synthetic protein corresponding to the entire *vpr* gene product from the human immunodeficiency virus HIV-1 is recognized by antibodies from HIV-infected patients

H. GRAS-MASSE, J.C. AMEISEN*, C. BOUTILLON, J.C. GESQUIÈRE, S. VIAN*, J.L. NEYRINCK*, H. DROBECQ, A. CAPRON*, and A. TARTAR

*Biomolecular Chemistry Facility, CNRS-1309, and *Centre of Immunology and Parasite Biology, INSERM U 167, Pasteur Institute of Lille, Lille, France*

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The 95 amino acid-protein encoded by the non-structural *vpr* gene of the human immunodeficiency virus type 1(LAV-1BRU isolate) was chemically synthesized by solid phase methodology. The sythetic *vpr* protein was characterized by amino acid analysis, sequence analysis, RP-HPLC, and urea-SDS PAGE. Using a radioimmunoassay, antibodies to the synthetic protein were detected in sera of 25% of HIV 1-seropositive patients tested. Western blot analysis suggested that the antibodies preferentially recognize the dimeric form of *vpr*.

*Key words*: HIV-1; HIV-infected patients; peptide synthesis; serological reactivity; solid phase; *vpr*

The human immunodeficiency virus 1 (HIV-1) is the etiologic agent of the acquired immunodeficiency syndrome (AIDS). Beside the three genes (*gag, pol, env*) that encode the structural proteins, the HIV-1 provirus encodes six additional functional genes (*tat, rev, nef, vif, vpu, vpr*). The non-structural proteins encoded by these six genes are absent from the virion but are expressed by the infected cell, and have an intricate regulatory role on the synthesis of infectious virions (1). Investigation of the precise function of these regulatory proteins and of the immune response they elicit may help in understanding the mechanisms involved in the maintenance of silent phases of HIV infection that can precede seroconversion (2, 3), and in the evolution from the asymptomatic seropositive phase of the infection towards AIDS.

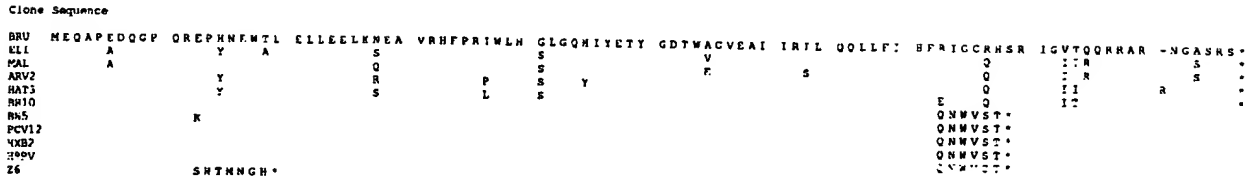
The *vpr* gene product has been described and investigated in only one study (4), and is the only non-structural protein whose function has remained unknown, although very recent preliminary data suggest that *vpr* might act as a transactivator (5). The *vpr* gene is highly conserved among different proviruses for which sequence information is available (6–14), although, in one infectious isolate (10), the *vpr* gene contains a stop codon after only 54 bases. Several strains contain an 18 or 19 amino acid *C*-terminal extension (11–14). This is the case for the LAV-1BRU sequence (11), which is the reference strain in Europe (Table 1). The hydrophilicity profile (15) of this sequence suggests that both the *N*- and *C*-terminal ends of the protein have a high probability of being major B cell epitopes (Fig. 1) implying that the whole structure might be of interest for immunological studies.

Using an incomplete *vpr* recombinant protein from the BH10/HIV-1 sequence lacking 8 *N*-terminal and 2 *C*-terminal amino acids and expressed in a fusion protein, Wong-Staal *et al.* (4) have detected antibodies to *vpr* in a third of sera from HIV-infected patients. Chemical synthesis of such a relatively small protein appeared as an attractive alternative to recombinant DNA technology, with regard to the possibility of obtaining the full length sequence, including the very *N*- and *C*-terminal amino acids that were absent in the fusion protein used by Wong-Staal *et al.* (4), and

Abbreviations: t-BOC, *tert.*-butyloxycarbonyl; BrZ, bromobenzyloxycarbonyl; cHex, cyclohexyl; MeBzl, 4-methyl-benzyl; Tos, *p*-toluenesulfonyl; For, formyl; DNP, dinitrophenyl; DCC, dicyclohexylcarbodiimide; HOBt, hydroxybenzotriazole; TFA, trifluoroacetic acid; DMF, dimethylformamide; DCM, dichloromethane; DIEA, diisopropylethylamine; HF, hydrofluoric acid; DNP; $PBu_3$, tributylphosphine; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.

TABLE 1

*Alignment of the HIV-1* vpr*-gene protein sequences*



BRU(7), ELI, MAL (8), ARV2 (9), HAT3 (10), BH10, BH5 (11), H9PV (12), BXB2 (13), PCV12 (14), Z6 (15).

The different sequences were aligned with reference to BRU. Only the amino acids differing from this sequence were indicated.

avoiding any contaminating protein from the bacterial host, which can lead to false positive serological results. Here we report the synthesis by solid phase methodology (16) of the entire 95-residue *vpr* protein derived from the LAV-1BRU isolate, with the purpose of providing sufficient material to enable extensive investigation of the immune response to *vpr* in HIV-infected patients, its immunogenicity in experimental models, and its possible biological function.

## MATERIALS AND METHODS

### *Synthesis and purification* vpr

Chemical synthesis was performed using a fully automated reprogrammed Applied Biosystems model 430 A peptide synthesizer. tBOC-N$\alpha$ protected amino acids (Peptide Institute, Osaka) were sequentially coupled to the tBOC-OBzl-Ser-$OCH_2$ PAM-resin (17) (Applied Biosystems) (0.5 mmol; loading of starting resin: 0.67 mmol per gram). Side chain protecting groups were: Asp (OcHex) (18), Glu (OcHex) (19), Ser(Bzl), Thr(Bzl), Arg(Tos), Tyr(BrZ), Cys(4-MeBzl), Trp(For), and His (DNP). Amino acids were coupled as symmetric anhydrides (20), except for Asn, Gln, Arg(Tos), and His(DNP), which were coupled according to the DCC/HOBt method (21). Systematic double-coupling (4 mmol amino acid per coupling) was performed, first coupling in DMF, and second coupling in DCM. Coupling times were 26 and 34 min for the first and second coupling as symmetric anhydride, progressively increased to 32 and 40 min respectively, 41 min for the DCC/HOBt method, progressively increased to 47 min. The programming was adapted to optimize vortexing during introduction of solvents and reactives. A part (25%) of the peptide resin, corresponding to peptide 46–95, was removed during the synthesis for further studies and to allow an adequate swelling of the residual resin. After assembly of the complete protected peptide chain, 3.9 g (67% yield) of peptide resin were obtained. The peptide resin (1 g) was treated three times with 15% mercaptoethanol-2% DIEA in DMF for 60 min each time, in order to remove the DNP groups from the histidine residues. The tBOC group was removed with 50% TFA, and the resin was dried, cleaved and deprotected in a Teflon-Kel F-HF apparatus (ASTI, Courbevoie, France), in low-concentration of HF in dimethylsulfide, in the presence of *p*-cresol and *p*-thiocresol (25:65:7.5:2.5) for 2 h at 0°, followed by a high-HF procedure in HF, *p*-cresol and thiocresol (90:7.5:2.5) for 1 h at 0° (22). The cleaved deprotected peptide was precipitated and washed with cold diethylether, and then dissolved in 5% acetic acid and lyophilized. The crude peptide (600 mg) was dissolved in neat TFA (30 mL) and was precipitated by pouring into dry ice-cooled diethylether (300 mL). After centrifugation, the precipitate was dissolved in water (20 mL), treated with 10 $\mu$L $PBu_3$ (40 $\mu$mol) (23) for 30 min and concentrated to 10 mL by ultrafiltration. The solution was dialyzed against 0.1% O-octyl-D glucopyranoside (Aldrich) in water (50 mL), then against 1% acetic acid (400 mL), on a YM5 (Amicon, Danvers, USA) membrane, and lyophilized, to give 260 mg protein (acetate), corresponding to 218 mg protein without counterions, as determined by hydrolysis and quantitative amino acid analysis (30% yield, based on starting tBOC-Ser(OBzl)-PAM resin).

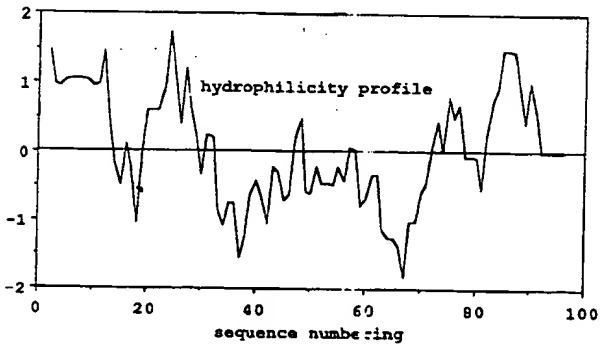



FIGURE 1

Hydrophilicity profile (15) of *vpr* gene product (LAV-1BRU sequence).

### *Hydrolysis of free peptides and peptide resins*

Dried samples of the peptide resin were hydrolyzed with 12 N HCl/propionic acid/phenol (5:5:1) for 2 h at 140° (24). Hydrolysis of the free peptides were with 6 N HCl/phenol (10:1) at 110° for 24, 48, or 72 h in an

evacuated sealed tube. Amino acids were quantitated on a Beckman amino acid analyzer model 7300 with ninhydrin detection.

*Analytical high-pressure liquid chromatography*
Purified synthetic *vpr* protein (5 μg/5 μL buffer A) was applied onto a Vydac C4 5μ 300 Å column (7.5 × 100 mm) in a Shimadzu system. Buffer A was 0.05% TFA in water, buffer B was 0.042% TFA, 75% acetonitrile in water; flow rate was 0.2 mL/min. Detection was by absorbance at 215 nm.

*Urea-SDS polyacrylamide gel electrophoresis*
Electrophoretic characterization of the synthetic *vpr* protein was according to Swank & Munkress (25) on 12.5% polyacrylamide gel prepared with the acrylamide/bis (acrylamide) ratio (10:1), and the inclusion of 8 M urea in the SDS containing gel buffer. The gel was run at 130 V, for about 10 h. Molecular weight markers (Pharmacia) were 17 200; 14 600; 8 240; 6 380; 2 560.

*Amino-terminal sequence determination*
A sample of purified synthetic *vpr* protein was loaded onto a polybrene-treated glass fiber disk, and the Edman degradation was performed for 30 cycles in an Applied Biosystems protein sequencer equipped with an on-line model 120 A PTH analyzer.

*Detection of anti-*vpr*-antibodies*
Sera from 23 HIV-1 infected patients and from 40 healthy controls were investigated. Seropositivity (antibodies to the HIV-1 structural proteins *gag, pol,* and *env*) was determined by an enzyme-linked immunosorbent assay (ELISA) (Elavia, Diagnostic Pasteur) and by Western blotting (Du Pont de Nemours). Patients were classified according to the CDC (Center for Disease Control, Atlanta) classification: stage II (asymptomatic), and stage IV (AIDS).

*Solid-phase radioimmunoassay (RIA)* was performed as previously described (2). Briefly, Star tubes (Nunc, Denmark) were coated at room temperature with 250 μL protein (2 μg) in 0.015 M carbonate-0.035 M bicarbonate buffered at pH 9.6. The proteins used as antigens were the chemically synthesized *vpr*, an *E. coli* recombinant *nef* protein, and an *E. coli* recombinant *p24* protein, purified to more than 97% and corresponding to the complete coding sequences of the LAV-1BRU isolate (provided by Transgene SA, Strasbourg, France). The tubes were saturated by incubation with 3% bovine serum albumin (BSA) in phosphate buffer saline (PBS) for 2 h at room temperature, washed twice in PBS, and then incubated overnight with human serum at a 1:50 dilution in PBS-0.3% BSA. After three washes in PBS, bound human antibodies were detected by overnight incubation at room temperature with $^{125}$I-radiolabelled murine monoclonal antibodies to human IgG1 (Seward, UK) in PBS-0.3% BSA, followed by three washes in PBS containing 0.01% Tween, and bound radioactivity was counted in a gamma counter (LKB, Sweden). In each assay, 10 different sera from healthy individuals were used as controls. The increase in bound radioactivity observed in the presence of positive sera ($B_x$) versus control sera ($B_o$) was calculated:

$$\Delta + \% = [100 - (B_o/B_x)100] \pm \text{SEM}$$

Sera were considered positive when superior to the cutoff (mean of control values + 3 SD).

*Western blot.* After urea-SDS electrophoresis, the synthetic protein was electroblotted to a nitrocellulose filter. Strips were saturated with PBS-milk 5% during 1 h at room temperature, then incubated with human serum at 1:100 dilution in PBS-milk 0.5% overnight at 4°. After 5 washes in PBS-Tween 0.3%, biotinylated polyclonal anti-human immunoglobulin antisera (Du Pont) were incubated at 1:150 dilution in PBS-milk 0.5% for 2 h at 4°. After 5 washes in PBS-Tween 0.3%, strips were saturated with peroxidase-avidin (Du Pont) at 1:150 dilution in PBS-milk 0.5% and revealed with the enzyme substrate (4-chloro-naphthol, Kirkegaard & Penny Lab, Inc.).

## RESULTS AND DISCUSSION

*Synthesis*
The synthesis followed the stepwise solid-phase strategy (16), with the acid-labile group *tert.*-butyloxycarbonyl (Boc) for temporary $N^{\alpha}$ protection, using a "PAM"-resin (17), able to withstand the 95 deprotection steps required for the synthesis. Cyclohexyl esters of *tert.*-butyloxycarbonyl aspartic (18) and glutamic acids (19) were used because they are more acid stable than corresponding benzyl esters, and minimize aspartimide and pyrrolidone carboxylic acid formation, respectively. Key to the high yield of our synthesis was the efficiency of coupling steps, using systematic double-coupling in two different solvents, and amino acids activated as the highly reactive symmetric anhydrides (20), prepared automatically before each step, without delay between neutralization and coupling step: this precaution is particularly useful after the Gln-Gln sequences in positions 84–86 and 64–65, and avoids the occurrence of a significant chain termination by cyclisation to pyroglutamic acid. At the end of the synthesis, the weight of the peptide resin corresponded to a global yield of 67%, or to an average yield of 99.6% per step. Progress in synthesis was followed by amino acid analysis of hydrolyzed samples of peptidyl-resin at several stages of chain-assembly. The distribution of residues made it possible to check the regular incorporation of the residues, considering Arg a marker for the *C*-terminal part of

TABLE 2

*Lanes 1–4: amino acid analysis of peptide resins*

| | 1 (82–95) resin 14 residues | | 2 (46–95) resin 50 residues | | 3 (26–95) resin 70 residues | | 4 (1–95) resin 95 residues | | 5 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Calc. | Theor. | Calc. | Theor. | Calc. | Theor. | Calc. | Theor. | Calc. | Theor. |
| Asp | 1.02 | 1 | 2.43 | 2 | 3.08 | 3 | 50.8 | 5 | 4.91 | 5 |
| Thr | 0.71 | 1 | 1.83 | 3 | 2.49 | 3 | 2.91 | 4 | 4.00 | 4 |
| Ser | 1.44 | 2 | 1.75 | 3 | 1.72 | 3 | 2.48 | 3 | 2.96 | 3 |
| Glu | 1.92 | 2 | 6.04 | 6 | 8.10 | 9 | 16.30 | 18 | 17.33 | 18 |
| Pro | | | | | 0.81 | 9 | 2.62 | 4 | 3.18 | 4 |
| Gly | 1.01 | 1 | 5.05 | 5 | 7.12 | 7 | 8.85 | 8 | 8.18 | 8 |
| Ala | 1.97 | 2 | 4.17 | 4 | 5.36 | 5 | 6.73 | 6 | 6.36 | 6 |
| Val | 0.94 | 1 | 1.94 | 2 | 2.69 | 3 | 3.23 | 3 | 3.15 | 3 |
| Trp | | 0 | ND | 1 | ND | 2 | ND | 3 | ND | 3 |
| Ile | | | 4.79 | 6 | 6.60 | 8 | 7.64 | 8 | 7.97 | 8 |
| Leu | | | 2.95 | 3 | 4.82 | 5 | 8.45 | 9 | 9.41 | 9 |
| Tyr | | | 1.62 | 2 | 1.58 | 2 | 1.72 | 2 | 2.13 | 2 |
| Phe | | | 1.88 | 2 | 2.95 | 3 | 3.18 | 3 | 3.18 | 3 |
| His | | 0 | ND | 2 | ND | 5 | ND | 6 | 5.25 | 6 |
| Lys | | | | | 1.01 | 1 | 0.78 | 1 | 0.95 | 1 |
| Arg | 3.61 | 4 | 8.72 | 8 | 10.27 | 9 | 12.16 | 11 | 10.90 | 11 |

The value 1 was calculated according to stable amino acid average values (Asp, Glu, Gly, Ala, Val, Ile, Leu, Phe, Lys, Arg). His(DNP) was not identified. Lane 5: amino acid analysis ND, not determined.

the molecule (8 Arg residues out of 11 are present among the 35 *C*-terminal amino acids), and Glu/Gln as a marker for the *N*-terminal part of the molecule (9 residues out of 18 are present in the 26 *N*-terminal amino acids). As shown in Table 2 lanes 1–4, the molar ratios of amino acids were close to the theoretical values in each sample, indicating the absence of extensive chain termination during the synthesis. Between the two last samplings, we could use phenylalanine as an internal standard to calculate the yield of incorporation of Glu/Gln during the last 25 steps of the synthesis: whereas the number of phenylalanine remained constant between these two steps, 9 Glu or Gln were introduced during the synthesis. The actual increase in Glu/Gln relative to Phe was 7.14; this value corresponds to a global yield of 79%, or an average yield of 99.11% per step. This result was in good agreement with the yield calculated on the basis of the weight of the final peptide resin: the weight of the peptide resin at the end of the synthesis corresponds to a global yield of 67%, or an average yield of 99.6% per step.

After coupling of the final amino acid residue, the DNP groups of the histidine residues were removed by thiolysis before deprotection and cleavage by the "low-high" HF procedure (22). Under these conditions, all the protecting groups were removed. Preliminary trials were performed to purify the product using HPLC (gel filtration in dissociating buffers containing 4 M guanidine HCl on a Bio-Sil TSK-250 column (Bio-Rad), hydrophobic interaction chromatography on an (alkyl)aspartimide HIC column (Nest group) using 1.8 M ammonium sulfate-0.1 M potassium phosphate pH 7 as starting buffer, RP-HPLC on a Vydac C4 column in water-acetonitrile 0.05% TFA, ionic exchange on a sulfoethyl aspartimide SCX column (Nes group) using a buffer system 0.2 M NaCl/potassium phosphate 50 mM pH 3.5: in each case, formation test missing of aggregates during chromatography was observed. Since highly resoluting chromatographic techniques could not be used, we chose the simplest accessible method to purify the synthetic protein; the crude product was first precipitated in TFA/diethylether to remove most of the residual non-volatile hydrophobic scavengers and by-products. The peptide was then redissolved in water and, using a dialysis membrane with a 5 000 molecular weight cutoff, was concentrated by ultrafiltration in reductive conditions (tributylphosphine), and purified by dialysis first in the presence of 0.1% of a non-ionic dialyzable detergent (O-octyl-D-glucopyranoside), then extensively against acidified water. Since capping was not performed during synthesis, and since purification by dialysis was not likely to have removed deletion peptides differing from *vpr* by a single missing internal amino acid, sequence analysis could be considered representative of the coupling efficiency during the last steps of chain elongation. The *N*-terminal sequence of the purified peptide was determined by 30 cycles of Edman degradation. The premature appearance ("preview") of amino acids during successive cycles of degradation is an indicator of the rate of deletions that occurred during synthesis. The cumulative level of preview was quantified from 28 sequencing runs (Table 3). Taking into account the limits of precision due to the increasing background, a cumulative preview of 19% was calculated, corresponding to an average preview of 0.6% per step.

TABLE 3

*Quantitative sequence analysis of synthetic* vpr

| Cycle No. | Residue | Cumulative preview (%) | Average preview per step (%) |
|---|---|---|---|
| 1 | Gln | 0.22 | 0.11 |
| 2 | Ala | 0.4 | 0.20 |
| 3 | Pro | 1.5 | 0.50 |
| 5 | Asp | 2.0 | 0.40 |
| 10 | Arg | 5.7 | 0.57 |
| 13 | Thr | 7.6 | 0.58 |
| 14 | Asn | 9.8 | 0.70 |
| 17 | Thr | 10 | 0.59 |
| 25 | Lys | 12 | 0.48 |
| 28 | Ala | 19 | 0.70 |

The premature appearance (preview) of amino acids was calculated as the ratio of peak area of amino acids *i* at cycle *i-1* to the sum of the peak area of amino acid *i-1* at cycle *i-1* and of the peak area of amino acid *i* at cycle *i*. All peak areas were corrected for background.

Considering this 0.6% deletion ratio as a mean value for 94 steps, we estimated that 56% of the chains had the target sequence, while 32% of the final product consisted of a mixture of closely related peptides with a single amino-acid deletion (26).

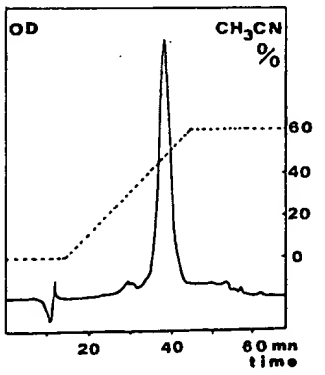



FIGURE 2

Analytical RP-HPLC. Purified synthetic *vpr* (5 μg/5 μL buffer A) was applied onto a Vydac C4 5 μ 300 Å column (7.5 × 100 mm). Buffer A was 0.5% TFA in water, buffer B was 0.5% TFA-75% acetonitrile in water; flow rate was 0.2 mL/mn. Detection was by absorbance at 215 nm, 0.64 AUFS.

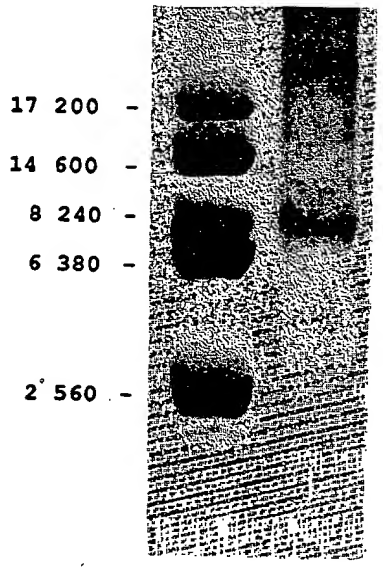



FIGURE 3

Urea-SDS gel electrophoresis on a 12.5% polyacrylamide gel containing 8 M urea (acrylamide/bis-acrylamide ratio: 10/1). Molecular weight markers (Pharmacia) are shown on the left lane for comparison and were: 17 200; 14 600; 8 240; 6 380; 2 560.

*Chemical characterization of* vpr

*Amino acid analysis* of the purified protein was performed after acid hydrolysis for 24, 48, and 72 h, in order to obtain an accurate determination of sterically hindered amino acids as well as sensitive amino acids. These analyses indicated that the purified protein had the expected amino acid composition (Table 2, lane 5).

*Analytical RP-HPLC* of the purified *vpr* performed on a C4 column showed a broad but single and symmetrical peak (Fig. 2). This profile was probably due to the formation of aggregates.

*Urea-SDS gel electrophoresis* was performed in conditions allowing an optimal resolution of peptides of molecular weight less than 10 000. In spite of the strong-dissociating conditions used, two major sharp bands were observed; the first one had an apparent molecular weight of 8 500, while the second band corresponded to the dimer (Fig. 3). When using stronger reductive conditions ($PBu_3$ instead of mercaptoethanol in the sampling buffer), it was possible to obtain the monomer only (data not shown).

*Detection of* vpr-*specific antibodies*

Sera from 23 HIV infected seropositive individuals were tested: 13 sera were from asymptomatic seropositive individuals (CDC stage II) and 10 from AIDS patients (CDC stage IV). Using a solid phase radioimmunoassay, antibodies to *vpr* were detected in 26% of the sera, namely 3 CDC stage II sera, and 3 (30%) from CDC stage IV sera; no antibody to *vpr* was detected in sera from 40 healthy controls. This frequency is in the reported range of the antibody prevalence to other regulatory gene products (*nef, vif*, and *tat*), that is, between 30 and 65% depending on the antigen (27, 28). Using the same assay and the same sera, the antibody response to *vpr* was compared to the antibody response to another regulatory gene product (*nef*, the negative regulatory factor). As shown in Table 4, fewer sera reacted with *vpr* than with *nef*. In contrast to the antibody response to *nef*, which was, as the antibody response to the virion core (gag) protein *p24*, less frequent at the terminal stage of the disease, the response to *vpr* did not significantly differ in sera from asymptomatic individuals and from AIDS patients, suggesting an absence of correlation with disease progression.

TABLE 4

*Prevalence of the antibody response to the regulatory gene products* vpr *and* nef *and to the virion core protein* p24 *in HIV 1-infected seropositive patients*

| | *vpr* | *nef* | *p24* |
|---|---|---|---|
| Healthy controls (40 sera) | 0% | 0% | 0% |
| CDC Stage II (13 sera) | 23% | 61% | 84% |
| CDC Stage IV (10 sera) | 30% | 30% | 40% |

The antibody response was measured in a solid phase radioimmunoassay. CDC (Center for Disease Control) stage II: asymptomatic; stage IV: AIDS.

Sera from 8 HIV-seropositive individuals were further analyzed using the Western-blot technique. Four sera reacted with the electroblotted protein (Fig. 4). Interestingly, although the monomeric form of *vpr* was predominant on the lanes, as shown after Coomassie blue or Amido black staining, antibodies preferentially recognized the dimeric form of *vpr* (Fig. 4B). This suggests that the dimeric form of *vpr* may correspond to the structure naturally exposed to the immune system during HIV-infection.

The antibody response to *vpr* is currently being analyzed in a larger population of HIV-1 infected patients, including HIV-1 infected seronegative individuals who present an antibody response to the *nef* regulatory gene product (3). The availability of large quantities of synthetic *vpr* will allow exploration of the T-cell response to this protein in HIV-infected patients and of its immunogenicity in animal models. Since a chemically synthesized HIV regulatory gene product (*tat*, 86 amino acids) has been shown to be taken up by cells, and to subsequently exert its transactivator effect on the HIV promoter (29), *vpr* func-

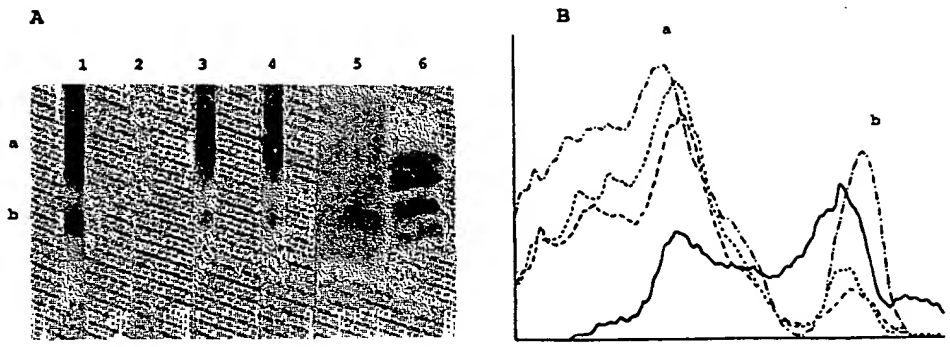



FIGURE 4

A: identification of antibodies to *vpr* by Western blot analysis in sera from four HIV-infected patients (lanes 1, 2, 3, 4). Molecular weight standards (lane 6) were as in Fig. 3. B: analysis with a video densitometer (Chromoscan 3 Joyce Loebl) of the lanes; a: *vpr* dimer, b: *vpr* monomer; — — amido black staining in the absence of any sera (lane 5); peroxidase staining after reaction with HIV positive sera: -·-·- lane 1; ····· lane 3; ---- lane 4. Horizontal axis: scan length (25 mm). Vertical axis: reflectivity (589 nm filter).

tion could be explored by testing the potential functional effect of synthetic *vpr* on infected and uninfected cells of the immune system.

## CONCLUSION

Although formation of exceptionally stable agregates, which may be related to the unusual hydrophobicity profile, precluded the complete purification of synthetic *vpr* by high performance chromatography techniques, the synthetic peptide corresponding to the entire *vpr* gene product of HIV should represent a valuable tool, exempt from any biologically significant contaminant, to investigate the immune response of HIV-infected patients.

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Address:

*H. Gras-Masse*
Institut Pasteur
1 rue Calmette
F59019 Lille cedex
France

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# Short Communication

# Serum Reactivity to HIV-1 Accessory Gene Products Distinguishes East African from West African HIV Strains as Infecting Agent

JAAP GOUDSMIT,[1] JOHN T. DEKKER,[1] CHARLES A.B. BOUCHER,[1] LIA SMIT,[1] ANTHONY DE RONDE,[1] CHRISTINE DEBOUCK,[2] and FRANCIS BARIN[3]

Sir, the accessory gene products of human immunodeficiency virus type 1 (HIV-1), *nef, tat, rev,* and *vpr,* show 31–36% (34, 31, 36, and 35%, respectively) amino acid identity with the homologous gene products of HIV-2, but *vpu* is unique to HIV-1[1,2] and *vpx* to HIV-2.[3,4] All the HIV-1 accessory gene products are known to be antigenic in natural HIV-1 infection, although seronegativity may occur.[2,5] The highest frequency of reactivity in natural HIV-1 infection is observed for *nef.*[2] In a series of homosexual men, hemophiliacs, and intravenous drug abusers in Europe who had seroconverted to HIV-1 *gag*- and *env*-encoded proteins expressed in a prokaryotic vector system, 89% had antibodies to *nef.*[5] When subsequently the antigenicity of two additional regulatory proteins, *rev* and *tat,* was examined in the group of homosexual men, *rev*-specific and *tat*-specific antibodies were found in 47% and 29% of the subjects, respectively.[6] A controversy has arisen about the existence of dual infections with HIV-1 and HIV-2, in West Africa in particular. Tedder and coworkers[7] demonstrated reaction of sera of HIV-2–infected patients with HIV-1 antigens using immunoblotting as the detection method. The reverse was also seen, although less frequently. Based on competitive EIAs specific for HIV-1 or HIV-2, they concluded that dual infection was not the cause of the extensive cross-reactivity, even to envelope glycoprotein antibodies. Similar observations were made by us using the radioimmunoprecipitation assay with metabolically labeled HIV-1– and HIV-2–infected cells as antigen (Fig. 1). However, the difference in intensity of the bands corresponding to the HIV-1 envelope glycoproteins gp160/120 and the HIV-2 envelope glycoproteins gp140/110 often made the antibody preference clear. No advantages or disadvantages were observed by using HIV-1 strains of African origin (RUT, lane c). To evaluate the role of antibody reactivity to the HIV-1 accessory gene products in type-specific HIV serology, proteins encoded for *nef, tat, rev, vpr,* and *vpu* were produced in *Escherichia coli* as galactokinase fusion products and used as antigen in an EIA.[8] In the first series RIPAs were performed in parallel to the EIAs for the accessory gene products. Of the seven exclusively HIV-2 reactive sera, five lacked reactivity to the HIV-1 accessory gene products. Remarkably, the two sera that showed reactivity to the HIV-1 envelope on RIPA were the only ones reactive to HIV-1 accessory gene products. These results suggest that viruses belonging to type 2 may be as diverse as viruses belonging to type 1, as has previously been suggested by Zagury and coworkers.[9]

[1]Human Retrovirus Laboratory, Academic Medical Center, Amsterdam, The Netherlands.
[2]Department of Molecular Genetics, Smith Kline and French Laboratories, King of Prussia, PA.
[3]Laboratoire de Virologie, CHRU, Hôpital Bretonneau, Tours Cedex, France.

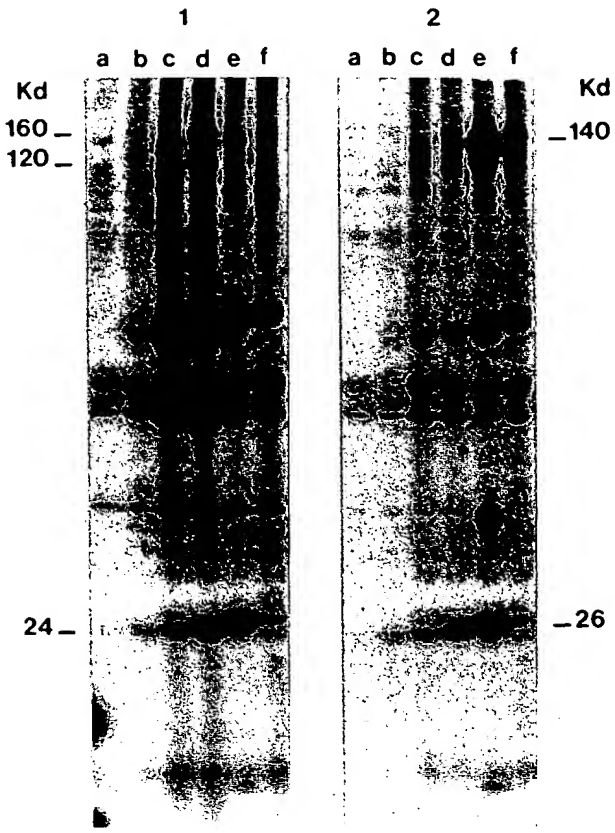


**FIG. 1.** Reactivity of a serum from an HIV-1–infected homosexual man (1) and from an HIV-2–infected woman (2) with antigens present in uninfected B cells (lane a), HIV-1 strain HTLV-IIIB (lane b), HIV-1 strain RUT (lane c), HIV-1 strain SF33 (lane d), HIV-2 strain LAV-2 ROD (lane e), and HIV-2 strain CBL 20 (lane f) infected B cells by radioimmunoprecipitation assay.

Subsequently, 24 sera of individuals originating from West Africa (Ivory Coast, Togo, Mali, Senegal, Guinea-Bissau, and Guinea), infected in West Africa, or partners and children of individuals of each of the former two categories were shown to react with a SIV peptide but not with an HIV-1 peptide previously shown to be discriminatory in a direct binding assay between HIV-1 and HIV-2.[10] Controls were 29 sera originating from an East African country (Tanzania) and shown to be reactive to HIV-1 antigens by a commercially available HIV-1 antibody assay using recombinant HIV-1 *gag* and *env* gene products as antigens (Abbott Labs, North Chicago, IL). Table 1 shows the frequency of reactivity to *nef, tat, rev, vpr,* and *vpu* gene products of HIV-1 in these two sets of African sera.

*Nef* bound European sera (89%) as well as East African sera (72%).[5] *Tat*- and *rev*-specific antibodies were seen more frequently in East Africans than in Europeans (83 versus 47% and 48 versus 29%, respectively).[6] No comparative data for *vpr* and *vpu* have been gathered as yet.

Reactivity to *vpr* and *vpu,* however, in our series of East Africans was highly frequent (66 and 45%). The frequencies of antibodies specific for *nef, tat, rev, vpr,* and *vpu* in West African sera were significantly lower, reactivities to *vpr* and *vpu* being totally absent (Table 1). Of the 24 sera, 9 (38%) from West Africa had reactivity to HIV-1 accessory gene products relative to 27 of the 29 (93%) East African sera.

In conclusion, reactivity to HIV accessory gene products may be useful in typing the infecting virus. Reactivity to the HIV-1 accessory gene products *vpr* and *vpu* indicates infection with an HIV type 1 strain. Reactivity to other accessory gene products may add to the identification of the infecting virus type but cannot be considered conclusive since cross-reactivity to HIV-1 accessory gene products of antibodies

TABLE 1. ANTIBODIES TO HIV-1 ACCESSORY GENE PRODUCTS IN SERA OF INDIVIDUALS FROM EAST AFRICA OR SERA OF INDIVIDUALS INFECTED BY HIV STRAINS ORIGINATING FROM WEST AFRICA

| *Serum origin* | *Number tested* | *Serum reactivity to* | | | | |
|---|---|---|---|---|---|---|
| | | nef | tat | rev | vpr | vpu |
| West Africa | 24 | 6 (25%) | 6 (25%) | 2 (8%) | 0 | 0 |
| East Africa | 29 | 21 (72%) | 24 (83%) | 14 (48%) | 19 (66%) | 13 (45%) |
| Fisher exact test results | | $p = 0.0007$ | $p = 0.00003$ | $p = 0.002$ | $p = 0.0000002$ | $p = 0.00008$ |

elicited by HIV-2 strains was documented. Antibodies to *vpr* and *vpu* were absent not only from all West African HIV-2 sera but also from 33 and 55%, respectively, of the East African HIV-1 sera, rendering impossible a distinction based solely on HIV-1 accessory gene product reactivity. The use of HIV-2 accessory gene products, especially *vpx*, may add to the discriminating power of the HIV-1 proteins described.

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Address reprint requests to:
*Jaap Goudsmit*
*AMC, Department of Virology, L1-157*
*Meibergdreef 15*
*1105, AZ Amsterdam*
*The Netherlands*

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# A synthetic protein corresponding to the entire *vpr* gene product from the human immunodeficiency virus HIV-1 is recognized by antibodies from HIV-infected patients

H. GRAS-MASSE, J.C. AMEISEN*, C. BOUTILLON, J.C. GESQUIÈRE, S. VIAN*, J.L. NEYRINCK*, H. DROBECQ, A. CAPRON*, and A. TARTAR

*Biomolecular Chemistry Facility, CNRS-1309, and *Centre of Immunology and Parasite Biology, INSERM U 167, Pasteur Institute of Lille, Lille, France*

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The 95 amino acid-protein encoded by the non-structural *vpr* gene of the human immunodeficiency virus type 1(LAV-1BRU isolate) was chemically synthesized by solid phase methodology. The sythetic *vpr* protein was characterized by amino acid analysis, sequence analysis, RP-HPLC, and urea-SDS PAGE. Using a radioimmunoassay, antibodies to the synthetic protein were detected in sera of 25% of HIV 1-seropositive patients tested. Western blot analysis suggested that the antibodies preferentially recognize the dimeric form of *vpr*.

*Key words*: HIV-1; HIV-infected patients; peptide synthesis; serological reactivity; solid phase; *vpr*

The human immunodeficiency virus 1 (HIV-1) is the etiologic agent of the acquired immunodeficiency syndrome (AIDS). Beside the three genes (*gag, pol, env*) that encode the structural proteins, the HIV-1 provirus encodes six additional functional genes (*tat, rev, nef, vif, vpu, vpr*). The non-structural proteins encoded by these six genes are absent from the virion but are expressed by the infected cell, and have an intricate regulatory role on the synthesis of infectious virions (1). Investigation of the precise function of these regulatory proteins and of the immune response they elicit may help in understanding the mechanisms involved in the maintenance of silent phases of HIV infection that can precede seroconversion (2, 3), and in the evolution from the asymptomatic seropositive phase of the infection towards AIDS.

The *vpr* gene product has been described and investigated in only one study (4), and is the only non-structural protein whose function has remained unknown, although very recent preliminary data suggest that *vpr* might act as a transactivator (5). The *vpr* gene is highly conserved among different proviruses for which sequence information is available (6–14), although, in one infectious isolate (10), the *vpr* gene contains a stop codon after only 54 bases. Several strains contain an 18 or 19 amino acid *C*-terminal extension (11–14). This is the case for the LAV-1BRU sequence (11), which is the reference strain in Europe (Table 1). The hydrophilicity profile (15) of this sequence suggests that both the *N*- and *C*-terminal ends of the protein have a high probability of being major B cell epitopes (Fig. 1) implying that the whole structure might be of interest for immunological studies.

Using an incomplete *vpr* recombinant protein from the BH10/HIV-1 sequence lacking 8 *N*-terminal and 2 *C*-terminal amino acids and expressed in a fusion protein, Wong-Staal *et al.* (4) have detected antibodies to *vpr* in a third of sera from HIV-infected patients. Chemical synthesis of such a relatively small protein appeared as an attractive alternative to recombinant DNA technology, with regard to the possibility of obtaining the full length sequence, including the very *N*- and *C*-terminal amino acids that were absent in the fusion protein used by Wong-Staal *et al.* (4), and

Abbreviations: t-BOC, *tert.*-butyloxycarbonyl; BrZ, bromobenzyloxycarbonyl; cHex, cyclohexyl; MeBzl, 4-methyl-benzyl; Tos, *p*-toluenesulfonyl; For, formyl; DNP, dinitrophenyl; DCC, dicyclohexylcarbodiimide; HOBt, hydroxybenzotriazole; TFA, trifluoroacetic acid; DMF, dimethylformamide; DCM, dichloromethane; DIEA, diisopropylethylamine; HF, hydrofluoric acid; DNP; $PBu_3$, tributylphosphine; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.

TABLE I

*Alignment of the HIV-1* vpr*-gene protein sequences*

| Clone | Sequence |
|---|---|
| BRU | MEQAPEDQGP QREPHNEWTL ELLEELKNEA VRHFPRIWLH GLGQHIYETY GDTWAGVEAI IRIL QQLLFI HFRIGCRHSR IGVTQQRRAR -NGASRS* |
| ELI | A Y A S S V Q IIR S . |
| MAL | A Q S E S Q I R S . |
| ARV2 | Y R P S Y Q I I R S . |
| HAT3 | Y S L S Q I I R . |
| BH10 | E Q I T . |
| BH5 | K QNWVST* |
| PCV12 | QNWVST* |
| HXB2 | QNWVST* |
| H9PV | QNWVST* |
| Z6 | SHTMNGH* QNWVST* |

BRU(7), ELI, MAL (8), ARV2 (9), HAT3 (10), BH10, BH5 (11), H9PV (12), HXB2 (13), PCV12 (14), Z6 (15).

The different sequences were aligned with reference to BRU. Only the amino acids differing from this sequence were indicated.

avoiding any contaminating protein from the bacterial host, which can lead to false positive serological results. Here we report the synthesis by solid phase methodology (16) of the entire 95-residue *vpr* protein derived from the LAV-1BRU isolate, with the purpose of providing sufficient material to enable extensive investigation of the immune response to *vpr* in HIV-infected patients, its immunogenicity in experimental models, and its possible biological function.

## MATERIALS AND METHODS

### *Synthesis and purification* vpr

Chemical synthesis was performed using a fully automated reprogrammed Applied Biosystems model 430 A peptide synthesizer. tBOC-Nα protected amino acids (Peptide Institute, Osaka) were sequentially coupled to the tBOC-OBzl-Ser-$OCH_2$ PAM-resin (17) (Applied Biosystems) (0.5 mmol; loading of starting resin: 0.67 mmol per gram). Side chain protecting groups were: Asp (OcHex) (18), Glu (OcHex) (19), Ser(Bzl), Thr(Bzl), Arg(Tos), Tyr(BrZ), Cys(4-MeBzl), Trp(For), and His (DNP). Amino acids were coupled as symmetric anhydrides (20), except for Asn, Gln, Arg(Tos), and His(DNP), which were coupled according to the DCC/HOBt method (21). Systematic double-coupling (4 mmol amino acid per coupling) was performed, first coupling in DMF, and second coupling in DCM. Coupling times were 26 and 34 min for the first and second coupling as symmetric anhydride, progressively increased to 32 and 40 min respectively, 41 min for the DCC/HOBt method, progressively increased to 47 min. The programming was adapted to optimize vortexing during introduction of solvents and reactives. A part (25%) of the peptide resin, corresponding to peptide 46–95, was removed during the synthesis for further studies and to allow an adequate swelling of the residual resin. After assembly of the complete protected peptide chain, 3.9 g (67% yield) of peptide resin were obtained. The peptide resin (1 g) was treated three times with 15% mercaptoethanol- 2% DIEA in DMF for 60 min each time, in order to remove the DNP groups from the histidine residues. The tBOC group was removed with 50% TFA, and the resin was dried, cleaved and deprotected in a Teflon-Kel F-HF apparatus (ASTI, Courbevoie, France), in low-concentration of HF in dimethylsulfide, in the presence of *p*-cresol and *p*-thiocresol (25:65:7.5:2.5) for 2 h at 0°, followed by a high-HF procedure in HF, *p*-cresol and thiocresol (90:7.5:2.5) for 1 h at 0° (22). The cleaved deprotected peptide was precipitated and washed with cold diethylether, and then dissolved in 5% acetic acid and lyophilized. The crude peptide (600 mg) was dissolved in neat TFA (30 mL) and was precipitated by pouring into dry ice-cooled diethylether (300 mL). After centrifugation, the precipitate was dissolved in water (20 mL), treated with 10 μL $PBu_3$ (40 μmol) (23) for 30 min and concentrated to 10 mL by ultrafiltration. The solution was dialyzed against 0.1% O-octyl-D glucopyranoside (Aldrich) in water (50 mL), then against 1% acetic acid (400 mL), on a YM5 (Amicon, Danvers, USA) membrane, and lyophilized, to give 260 mg protein (acetate), corresponding to 218 mg protein without counterions, as determined by hydrolysis and quantitative amino acid analysis (30% yield, based on starting tBOC-Ser(OBzl)-PAM resin).

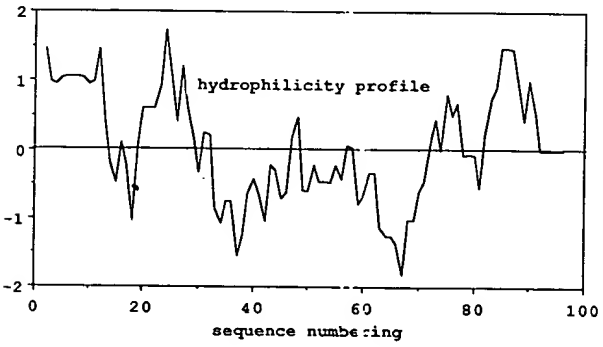


FIGURE I

Hydrophilicity profile (15) of *vpr* gene product (LAV-1BRU sequence).

### *Hydrolysis of free peptides and peptide resins*

Dried samples of the peptide resin were hydrolyzed with 12 N HCl/propionic acid/phenol (5:5:1) for 2 h at 140° (24). Hydrolysis of the free peptides were with 6 N HCl/phenol (10:1) at 110° for 24, 48, or 72 h in an

evacuated sealed tube. Amino acids were quantitated on a Beckman amino acid analyzer model 7300 with ninhydrin detection.

*Analytical high-pressure liquid chromatography*
Purified synthetic *vpr* protein (5 μg/5 μL buffer A) was applied onto a Vydac C4 5μ 300 Å column (7.5 × 100 mm) in a Shimadzu system. Buffer A was 0.05% TFA in water, buffer B was 0.042% TFA, 75% acetonitrile in water; flow rate was 0.2 mL/min. Detection was by absorbance at 215 nm.

*Urea-SDS polyacrylamide gel electrophoresis*
Electrophoretic characterization of the synthetic *vpr* protein was according to Swank & Munkress (25) on 12.5% polyacrylamide gel prepared with the acrylamide/bis (acrylamide) ratio (10:1), and the inclusion of 8 M urea in the SDS containing gel buffer. The gel was run at 130 V, for about 10 h. Molecular weight markers (Pharmacia) were 17 200; 14 600; 8 240; 6 380; 2 560.

*Amino-terminal sequence determination*
A sample of purified synthetic *vpr* protein was loaded onto a polybrene-treated glass fiber disk, and the Edman degradation was performed for 30 cycles in an Applied Biosystems protein sequencer equipped with an on-line model 120 A PTH analyzer.

*Detection of anti-*vpr*-antibodies*
Sera from 23 HIV-1 infected patients and from 40 healthy controls were investigated. Seropositivity (antibodies to the HIV-1 structural proteins *gag, pol,* and *env*) was determined by an enzyme-linked immunosorbent assay (ELISA) (Elavia, Diagnostic Pasteur) and by Western blotting (Du Pont de Nemours). Patients were classified according to the CDC (Center for Disease Control, Atlanta) classification: stage II (asymptomatic), and stage IV (AIDS).

*Solid-phase radioimmunoassay (RIA)* was performed as previously described (2). Briefly, Star tubes (Nunc, Denmark) were coated at room temperature with 250 μL protein (2 μg) in 0.015 M carbonate-0.035 M bicarbonate buffered at pH 9.6. The proteins used as antigens were the chemically synthesized *vpr*, an *E. coli* recombinant *nef* protein, and an *E. coli* recombinant *p24* protein, purified to more than 97% and corresponding to the complete coding sequences of the LAV-1BRU isolate (provided by Transgene SA, Strasbourg, France). The tubes were saturated by incubation with 3% bovine serum albumin (BSA) in phosphate buffer saline (PBS) for 2 h at room temperature, washed twice in PBS, and then incubated overnight with human serum at a 1:50 dilution in PBS-0.3% BSA. After three washes in PBS, bound human antibodies were detected by overnight incubation at room temperature with $^{125}$I-radiolabelled murine monoclonal antibodies to human IgG1 (Seward, UK) in PBS-0.3% BSA, followed by three washes in PBS containing 0.01% Tween, and bound radioactivity was counted in a gamma counter (LKB, Sweden). In each assay, 10 different sera from healthy individuals were used as controls. The increase in bound radioactivity observed in the presence of positive sera ($B_x$) versus control sera ($B_o$) was calculated:

$$\Delta + \% = [100 - (B_o/B_x)100] \pm \text{SEM}$$

Sera were considered positive when superior to the cutoff (mean of control values + 3 SD).

*Western blot.* After urea-SDS electrophoresis, the synthetic protein was electroblotted to a nitrocellulose filter. Strips were saturated with PBS-milk 5% during 1 h at room temperature, then incubated with human serum at 1:100 dilution in PBS-milk 0.5% overnight at 4°. After 5 washes in PBS-Tween 0.3%, biotinylated polyclonal anti-human immunoglobulin antisera (Du Pont) were incubated at 1:150 dilution in PBS-milk 0.5% for 2 h at 4°. After 5 washes in PBS-Tween 0.3%, strips were saturated with peroxidase-avidin (Du Pont) at 1:150 dilution in PBS-milk 0.5% and revealed with the enzyme substrate (4-chloro-naphthol, Kirkegaard & Penny Lab, Inc.).

## RESULTS AND DISCUSSION

*Synthesis*
The synthesis followed the stepwise solid-phase strategy (16), with the acid-labile group *tert.*-butyloxycarbonyl (Boc) for temporary $N^{\alpha}$ protection, using a "PAM"-resin (17), able to withstand the 95 deprotection steps required for the synthesis. Cyclohexyl esters of *tert.*-butyloxycarbonyl aspartic (18) and glutamic acids (19) were used because they are more acid stable than corresponding benzyl esters, and minimize aspartimide and pyrrolidone carboxylic acid formation, respectively. Key to the high yield of our synthesis was the efficiency of coupling steps, using systematic double-coupling in two different solvents, and amino acids activated as the highly reactive symmetric anhydrides (20), prepared automatically before each step, without delay between neutralization and coupling step: this precaution is particularly useful after the Gln-Gln sequences in positions 84–86 and 64–65, and avoids the occurrence of a significant chain termination by cyclisation to pyroglutamic acid. At the end of the synthesis, the weight of the peptide resin corresponded to a global yield of 67%, or to an average yield of 99.6% per step. Progress in synthesis was followed by amino acid analysis of hydrolyzed samples of peptidyl-resin at several stages of chain-assembly. The distribution of residues made it possible to check the regular incorporation of the residues, considering Arg a marker for the *C*-terminal part of

TABLE 2

*Lanes 1–4: amino acid analysis of peptide resins*

| | 1 (82–95) resin 14 residues | | 2 (46–95) resin 50 residues | | 3 (26–95) resin 70 residues | | 4 (1–95) resin 95 residues | | 5 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Calc. | Theor. | Calc. | Theor. | Calc. | Theor. | Calc. | Theor. | Calc. | Theor. |
| Asp | 1.02 | 1 | 2.43 | 2 | 3.08 | 3 | 50.8 | 5 | 4.91 | 5 |
| Thr | 0.71 | 1 | 1.83 | 3 | 2.49 | 3 | 2.91 | 4 | 4.00 | 4 |
| Ser | 1.44 | 2 | 1.75 | 3 | 1.72 | 3 | 2.48 | 3 | 2.96 | 3 |
| Glu | 1.92 | 2 | 6.04 | 6 | 8.10 | 9 | 16.30 | 18 | 17.33 | 18 |
| Pro | | | | | 0.81 | 9 | 2.62 | 4 | 3.18 | 4 |
| Gly | 1.01 | 1 | 5.05 | 5 | 7.12 | 7 | 8.85 | 8 | 8.18 | 8 |
| Ala | 1.97 | 2 | 4.17 | 4 | 5.36 | 5 | 6.73 | 6 | 6.36 | 6 |
| Val | 0.94 | 1 | 1.94 | 2 | 2.69 | 3 | 3.23 | 3 | 3.15 | 3 |
| Trp | | 0 | ND | 1 | ND | 2 | ND | 3 | ND | 3 |
| Ile | | | 4.79 | 6 | 6.60 | 8 | 7.64 | 8 | 7.97 | 8 |
| Leu | | | 2.95 | 3 | 4.82 | 5 | 8.45 | 9 | 9.41 | 9 |
| Tyr | | | 1.62 | 2 | 1.58 | 2 | 1.72 | 2 | 2.13 | 2 |
| Phe | | | 1.88 | 2 | 2.95 | 3 | 3.18 | 3 | 3.18 | 3 |
| His | | 0 | ND | 2 | ND | 5 | ND | 6 | 5.25 | 6 |
| Lys | | | | | 1.01 | 1 | 0.78 | 1 | 0.95 | 1 |
| Arg | 3.61 | 4 | 8.72 | 8 | 10.27 | 9 | 12.16 | 11 | 10.90 | 11 |

The value 1 was calculated according to stable amino acid average values (Asp, Glu, Gly, Ala, Val, Ile, Leu, Phe, Lys, Arg). His(DNP) was not identified. Lane 5: amino acid analysis ND, not determined.

the molecule (8 Arg residues out of 11 are present among the 35 *C*-terminal amino acids), and Glu/Gln as a marker for the *N*-terminal part of the molecule (9 residues out of 18 are present in the 26 *N*-terminal amino acids). As shown in Table 2 lanes 1–4, the molar ratios of amino acids were close to the theoretical values in each sample, indicating the absence of extensive chain termination during the synthesis. Between the two last samplings, we could use phenylalanine as an internal standard to calculate the yield of incorporation of Glu/Gln during the last 25 steps of the synthesis: whereas the number of phenylalanine remained constant between these two steps, 9 Glu or Gln were introduced during the synthesis. The actual increase in Glu/Gln relative to Phe was 7.14; this value corresponds to a global yield of 79%, or an average yield of 99.11% per step. This result was in good agreement with the yield calculated on the basis of the weight of the final peptide resin: the weight of the peptide resin at the end of the synthesis corresponds to a global yield of 67%, or an average yield of 99.6% per step.

After coupling of the final amino acid residue, the DNP groups of the histidine residues were removed by thiolysis before deprotection and cleavage by the "low-high" HF procedure (22). Under these conditions, all the protecting groups were removed. Preliminary trials were performed to purify the product using HPLC (gel filtration in dissociating buffers containing 4 M guanidine HCl on a Bio-Sil TSK-250 column (Bio-Rad), hydrophobic interaction chromatography on an (alkyl)aspartimide HIC column (Nest group) using 1.8 M ammonium sulfate-0.1 M potassium phosphate pH 7 as starting buffer, RP-HPLC on a Vydac C4 column in water-acetonitrile 0.05% TFA, ionic exchange on a sulfoethyl aspartimide SCX column (Nes group) using a buffer system 0.2 M NaCl/potassium phosphate 50 mM pH 3.5: in each case, formation test missing of aggregates during chromatography was observed. Since highly resoluting chromatographic techniques could not be used, we chose the simplest accessible method to purify the synthetic protein; the crude product was first precipitated in TFA/diethylether to remove most of the residual non-volatile hydrophobic scavengers and by-products. The peptide was then redissolved in water and, using a dialysis membrane with a 5000 molecular weight cutoff, was concentrated by ultrafiltration in reductive conditions (tributylphosphine), and purified by dialysis first in the presence of 0.1% of a non-ionic dialyzable detergent (O-octyl-D-glucopyranoside), then extensively against acidified water. Since capping was not performed during synthesis, and since purification by dialysis was not likely to have removed deletion peptides differing from *vpr* by a single missing internal amino acid, sequence analysis could be considered representative of the coupling efficiency during the last steps of chain elongation. The *N*-terminal sequence of the purified peptide was determined by 30 cycles of Edman degradation. The premature appearance ("preview") of amino acids during successive cycles of degradation is an indicator of the rate of deletions that occurred during synthesis. The cumulative level of preview was quantified from 28 sequencing runs (Table 3). Taking into account the limits of precision due to the increasing background, a cumulative preview of 19% was calculated, corresponding to an average preview of 0.6% per step.

TABLE 3

*Quantitative sequence analysis of synthetic* vpr

| Cycle No. | Residue | Cumulative preview (%) | Average preview per step (%) |
|---|---|---|---|
| 1 | Gln | 0.22 | 0.11 |
| 2 | Ala | 0.4 | 0.20 |
| 3 | Pro | 1.5 | 0.50 |
| 5 | Asp | 2.0 | 0.40 |
| 10 | Arg | 5.7 | 0.57 |
| 13 | Thr | 7.6 | 0.58 |
| 14 | Asn | 9.8 | 0.70 |
| 17 | Thr | 10 | 0.59 |
| 25 | Lys | 12 | 0.48 |
| 28 | Ala | 19 | 0.70 |

The premature appearance (preview) of amino acids was calculated as the ratio of peak area of amino acids *i* at cycle *i-1* to the sum of the peak area of amino acid *i-1* at cycle *i-1* and of the peak area of amino acid *i* at cycle *i*. All peak areas were corrected for background.

Considering this 0.6% deletion ratio as a mean value for 94 steps, we estimated that 56% of the chains had the target sequence, while 32% of the final product consisted of a mixture of closely related peptides with a single amino-acid deletion (26).

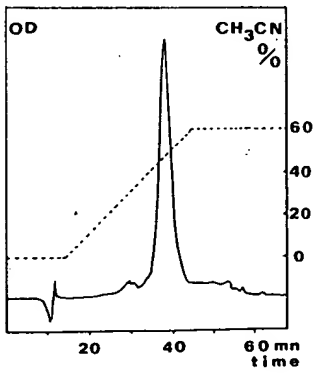


FIGURE 2

Analytical RP-HPLC. Purified synthetic *vpr* (5 μg/5 μL buffer A) was applied onto a Vydac C4 5 μ 300 Å column (7.5 × 100 mm). Buffer A was 0.5% TFA in water, buffer B was 0.5% TFA-75% acetonitrile in water; flow rate was 0.2 mL/mn. Detection was by absorbance at 215 nm, 0.64 AUFS.

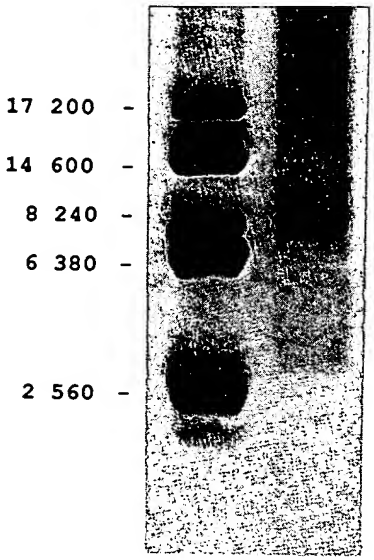


FIGURE 3

Urea-SDS gel electrophoresis on a 12.5% polyacrylamide gel containing 8 M urea (acrylamide/bis-acrylamide ratio: 10/1). Molecular weight markers (Pharmacia) are shown on the left lane for comparison and were: 17 200; 14 600; 8 240; 6 380; 2 560.

*Chemical characterization of* vpr

*Amino acid analysis* of the purified protein was performed after acid hydrolysis for 24, 48, and 72 h, in order to obtain an accurate determination of sterically hindered amino acids as well as sensitive amino acids. These analyses indicated that the purified protein had the expected amino acid composition (Table 2, lane 5).

*Analytical RP-HPLC* of the purified *vpr* performed on a C4 column showed a broad but single and symmetrical peak (Fig. 2). This profile was probably due to the formation of aggregates.

*Urea-SDS gel electrophoresis* was performed in conditions allowing an optimal resolution of peptides of molecular weight less than 10 000. In spite of the strong-dissociating conditions used, two major sharp bands were observed; the first one had an apparent molecular weight of 8 500, while the second band corresponded to the dimer (Fig. 3). When using stronger reductive conditions ($PBu_3$ instead of mercaptoethanol in the sampling buffer), it was possible to obtain the monomer only (data not shown).

*Detection of* vpr-*specific antibodies*

Sera from 23 HIV infected seropositive individuals were tested: 13 sera were from asymptomatic seropositive individuals (CDC stage II) and 10 from AIDS patients (CDC stage IV). Using a solid phase radioimmunoassay, antibodies to *vpr* were detected in 26% of the sera, namely 3 CDC stage II sera, and 3 (30%) from CDC stage IV sera; no antibody to *vpr* was detected in sera from 40 healthy controls. This frequency is in the reported range of the antibody prevalence to other regulatory gene products (*nef, vif*, and *tat*), that is, between 30 and 65% depending on the antigen (27, 28). Using the same assay and the same sera, the antibody response to *vpr* was compared to the antibody response to another regulatory gene product (*nef*, the negative regulatory factor). As shown in Table 4, fewer sera reacted with *vpr* than with *nef*. In contrast to the antibody response to *nef*, which was, as the antibody response to the virion core (gag) protein *p24*, less frequent at the terminal stage of the disease, the response to *vpr* did not significantly differ in sera from asymptomatic individuals and from AIDS patients, suggesting an absence of correlation with disease progression.

TABLE 4

*Prevalence of the antibody response to the regulatory gene products* vpr *and* nef *and to the virion core protein* p24 *in HIV 1-infected seropositive patients*

| | *vpr* | *nef* | *p24* |
|---|---|---|---|
| Healthy controls (40 sera) | 0% | 0% | 0% |
| CDC Stage II (13 sera) | 23% | 61% | 84% |
| CDC Stage IV (10 sera) | 30% | 30% | 40% |

The antibody response was measured in a solid phase radioimmunoassay. CDC (Center for Disease Control) stage II: asymptomatic; stage IV: AIDS.

Sera from 8 HIV-seropositive individuals were further analyzed using the Western-blot technique. Four sera reacted with the electroblotted protein (Fig. 4). Interestingly, although the monomeric form of *vpr* was predominant on the lanes, as shown after Coomassie blue or Amido black staining, antibodies preferentially recognized the dimeric form of *vpr* (Fig. 4B). This suggests that the dimeric form of *vpr* may correspond to the structure naturally exposed to the immune system during HIV-infection.

The antibody response to *vpr* is currently being analyzed in a larger population of HIV-1 infected patients, including HIV-1 infected seronegative individuals who present an antibody response to the *nef* regulatory gene product (3). The availability of large quantities of synthetic *vpr* will allow exploration of the T-cell response to this protein in HIV-infected patients and of its immunogenicity in animal models. Since a chemically synthesized HIV regulatory gene product (*tat*, 86 amino acids) has been shown to be taken up by cells, and to subsequently exert its transactivator effect on the HIV promoter (29), *vpr* func-

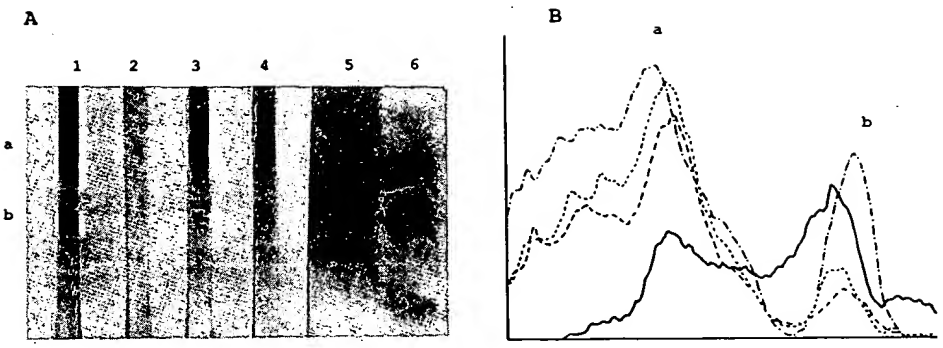


FIGURE 4

**A**: identification of antibodies to *vpr* by Western blot analysis in sera from four HIV-infected patients (lanes 1, 2, 3, 4). Molecular weight standards (lane 6) were as in Fig. 3. **B**: analysis with a video densitometer (Chromoscan 3 Joyce Loebl) of the lanes; **a**: *vpr* dimer, **b**: *vpr* monomer; —— amido black staining in the absence of any sera (lane 5); peroxidase staining after reaction with HIV positive sera: -·-·- lane 1; ····· lane 3; ---- lane 4. Horizontal axis: scan length (25 mm). Vertical axis: reflectivity (589 nm filter).

tion could be explored by testing the potential functional effect of synthetic *vpr* on infected and uninfected cells of the immune system.

## CONCLUSION

Although formation of exceptionally stable agregates, which may be related to the unusual hydrophobicity profile, precluded the complete purification of synthetic *vpr* by high performance chromatography techniques, the synthetic peptide corresponding to the entire *vpr* gene product of HIV should represent a valuable tool, exempt from any biologically significant contaminant, to investigate the immune response of HIV-infected patients.

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Address:

*H. Gras-Masse*
Institut Pasteur
1 rue Calmette
F59019 Lille cedex
France

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DRUG 281

# Antiviral Therapy in Human Immunodeficiency Virus Infections

## Current Status (Part II)†

*Eric Sandström*[1] and *Bo Öberg*[2]

1 Department of Dermatovenerology, Södersjukhuset, Stockholm, Sweden
2 Medivir AB, Huddinge, Sweden

### *Contents*



† Part I of this article appeared in the previous issue of the Journal.

**Summary** Part I of this article reviewed the targets against which anti-HIV drugs can be directed, problems in assessing active compounds (e.g. resistance development and use of surrogate end-points), and nucleoside analogues effective against HIV reverse transcriptase.

Intensive research is currently being undertaken in laboratories and hospitals to design and evaluate new inhibitors of HIV. In this work, combining different drugs is one important approach, both to decrease toxicity and to offset the rate of resistance development, which seems to be a major problem associated with therapy directed against the ever-changing HIV.

Therapeutic vaccines and immunomodulators are other modalities being actively evaluated against HIV and AIDS, although this effort has not yet yielded any licensed treatment.

It appears likely that new antiviral drugs and immunotherapies will be forthcoming during the next 5 years, that they will be used in a variety of combinations, and that the treatment options available for opportunistic infections will increase. These developments should improve the survival and the quality of life of patients with HIV infection.

## *6. Non-Nucleoside Reverse Transcriptase Inhibitors in Clinical Evaluation*

Several very selective, non-nucleoside, direct inhibitors of HIV-1 reverse transcriptase have been identified. These compounds have not been inhibitory to HIV-2 reverse transcriptase or other viral or cellular polymerases. Despite large differences in chemical structures, these compounds seem to bind at the same or at closely related sites on the enzyme. This similarity in action is also seen as a crosswise resistance between these compounds. This is their main weakness, and considerable efforts to overcome this problem are being made. The first reported compound of this type is TIBO [tetrahydro-imidazo- (4,5,1-*jk*)-(1,4)-benzodiazepin-2-(I,H)-one] and the structures of these compounds have been benzodiazepines, α-anilinophenyls, pyridones and piperazines.

### 6.1 TIBO and Thione Derivatives

The prototype TIBO compound R-14458 was found by Pauwels et al. (1990), and several compounds with improved activity were made. The first TIBO compound to be tested in patients was R-82913 (9-chloro-TIBO) [fig. 4].

The mechanism of action of TIBO compounds was found to be noncompetitive inhibition with respect to deoxynucleoside triphosphates, and an uncompetitive inhibition with respect to primer/template of HIV-1 reverse transcriptase (Debyser et al. 1991).

In cell cultures, R-82913 inhibited HIV-1 by 50% at 1.5 nmol/L to 0.65 μmol/L, depending on the virus isolate and assay method (Pauwels et al. 1990; White et al. 1991). HIV-2 was not inhibited at 310 μmol/L, and cellular toxicity was low at 31 to >870 μmol/L. These compounds showed a very high selectivity against HIV-1.

Pharmacokinetic studies with R-82913 in patients showed that the oral absorption was low, but nevertheless sufficient to achieve inhibitory plasma concentrations; $t_{1/2}$ was 3 days (De Wit et al. 1991). A peak plasma concentration of approximately 65 nmol/L occurred after a 200mg oral dose (AmFar 1992).

A phase I study in 22 patients with AIDS was performed using intravenous infusions of R-82913 (Pialoux et al. 1991). The dosage was escalated from 10 to 120 or 300 mg/day. Apart from a transient decrease in p24 levels, no clear antiviral effect was found and there was very rapid development of resistance (AmFar 1992). R-82913 was well tolerated and no severe adverse effects were reported.

### 6.2 α-Anilinophenylacetamide Derivatives

Little has been reported so far on this series of non-nucleoside reverse transcriptase inhibitors, but they are active against HIV-1 in the μg/L concen-

tration range in cell cultures, with a mechanism of action similar to that of TIBO.

A few of the α-anilinophenylacetamide compounds have been evaluated for oral absorption in healthy male volunteers. Eight hours after oral administration of 100mg of the lead compound R-18893, the plasma concentration was equal to the $IC_{50}$ in cell culture (i.e. 11 μg/L). R-89439 at the same dosage and time gave a plasma concentration 30 times the $IC_{50}$ (1.7 μg/L), and has been selected for further clinical evaluation.

The presently available clinical data have been reported for R-18893. At oral dosages of 200 to 1000mg 3 times daily for 3 months, no antiviral effects were reported, and some side effects occurred (Colebunders et al. 1992; De Brabander et al. 1992; De Cree et al. 1992). However, plasma concentrations may have been too low because of poor absorption, and resistance is likely to develop rapidly. It remains to be seen if this can be overcome with the improved compound R-89439.

### 6.3 Pyridone Derivatives

A series of pyridone derivatives were found to be selective HIV-1 reverse transcriptase inhibitors active in a manner similar to that of TIBO (Goldman et al. 1991, 1992). L-697661 [3-([(4,7-dichloro-1,3-benzoxazol-9-yl)methyl]amino)-5-ethyl-6-methylpyridin-2(1H)-one] (fig. 4) is a noncompetitive reverse transcriptase inhibitor with respect to deoxyguanine triphosphate (dGTP) and with respect to poly-rC.oligo-dG template primer. Phosphonoformate has been shown to be able to displace radiolabelled L-697639 (a compound similar to L-697661) from reverse transcriptase (Goldman et al. 1991), but it is not clear if the binding sites overlap or interact allosterically. The $IC_{50}$ of L-697661 against HIV-1 in cell culture was 0.012 to 0.2 μmol/L depending on virus and cell, and zidovudine-resistant virus was found to be sensitive. A synergistic effect of pyridone derivatives and either zidovudine or didanosine against HIV-1 has been seen in cell cultures.

HIV-1 selected for resistance to L-697639 had mutations at amino acid 103 (Lys to Asn) and 181 (Tyr to Cys) in reverse transcriptase, and was also cross-resistant to TIBO and nevirapine, but sensitive to zidovudine and didanosine (Nunberg et al. 1991).

Clinical evaluation of L-697661 in patients with HIV infection and/or AIDS has shown a dose-dependent decrease in p24 levels after 1 week of

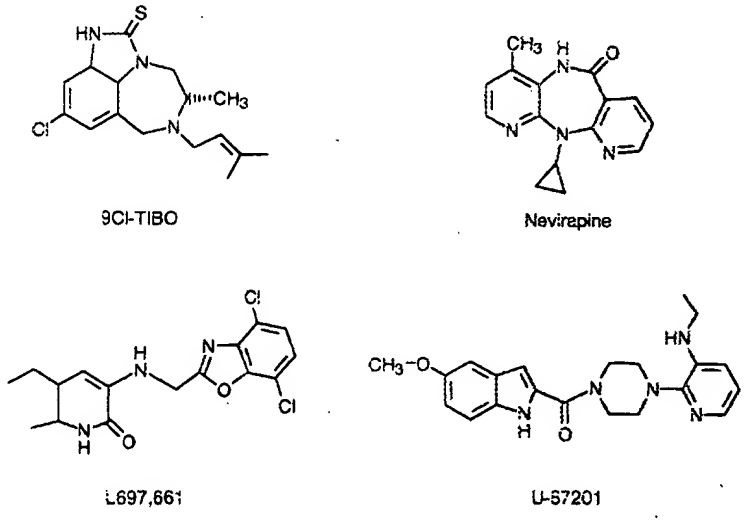



Fig. 4. Non-nucleoside reverse transcriptase inhibitors in clinical evaluation against HIV infection.

treatment with 25 to 500mg orally twice daily, but this effect was lost after 6 weeks coincident with the appearance of resistant virus (Kappes et al. 1992; Saag et al. 1992). This finding resulted in termination of the trials with monotherapy, but combination studies continue. The treatment was well tolerated but some increases in transaminases were noted. A peak plasma concentration of 2.56 μmol/L was observed after a 200mg oral dose (Davey et al. 1991).

### 6.4 *Bis* (Heteroaryl) Piperazines

*Bis* (heteroaryl) piperazines (BHAP compounds) [fig. 4] are specific HIV-1 reverse transcriptase inhibitors with a similar spectrum of activity as other non-nucleoside reverse transcriptase inhibitors. They were first reported by Romero et al. (1991) and were found to inhibit HIV-1 replication with nearly the same potency as zidovudine, and to have low cellular toxicity. Zidovudine-resistant HIV-1 was sensitive to BHAP but HIV-2 was not.

Poppe et al. (1992) demonstrated that resistance to BHAP compounds develops rapidly in cell culture. The mutation(s) responsible appear to differ from those causing resistance to other non-nucleoside reverse transcriptase inhibitors. Recombinant HIV-1 reverse transcriptase carrying mutations at 181 (Tyr to Cys) is less resistant to BHAP than to TIBO, and mutations at 228 (Leu to Phe) and 236 (Pro to Leu) caused resistance to BHAP but were sensitive to nevirapine and partly sensitive to L697661.

In 3 of 8 SCIDhu mice, BHAP prevented HIV-1 infection at a dosage of 200 mg/kg given as 2 daily oral doses. Pharmacokinetic studies in rats and mice indicate a good oral uptake and penetration into the CNS (Anstadt et al. 1991; Romero et al. 1991).

Clinical evaluation of one BHAP compound, U87201E, showed nonlinear pharmacokinetics after oral administration of 200, 400 and 600mg every 6 hours in healthy male volunteers. Serum concentrations above 30 μmol/L after administration of 400mg every 6 hours for 8 days caused increased bilirubin levels (Cox et al. 1992). U87201E 200mg every 6 hours was generally well tolerated after 2 weeks, with some elevated liver enzyme changes in women but not in men (Batts et al. 1992).

Efficacy data from clinical trials or clinical development of resistance (which is expected to occur rapidly) have not yet been reported.

### 6.5 Nevirapine

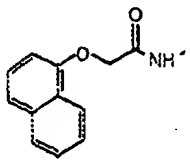
Nevirapine (fig. 4) was first described by Merluzzi et al. (1990) as a selective inhibitor of HIV-1 reverse transcriptase. It is a noncompetitive inhibitor with respect to dGTP. At concentrations of about 0.04 μmol/L nevirapine inhibits HIV-1 in cell cultures, and is toxic to cells at 321 μmol/L. Both from photoaffinity work (Cohen et al. 1991; Wu et al. 1991) and from the development of resistant mutants in cell cultures (Grob et al. 1992; Richman et al. 1991), it is clear that nevirapine interacts with Tyr 181 and Tyr 188 in the HIV-1 reverse transcriptase.

Nevirapine is synergistic with zidovudine, active against zidovudine-resistant HIV-1, and inactive against HIV-1 resistant to TIBO or L-693593 (Nunberg et al. 1991; Richman 1992). Its mechanism of action is thus similar to that of TIBO and other non-nucleoside inhibitors selective for HIV-1 reverse transcriptase.

Clinical evaluation of nevirapine alone and in combination with zidovudine has shown a dose-related reduction in p24 antigenaemia with dosages of 12.5 to 400mg orally 8-hourly (Cheeseman 1992). However, reduction in p24 levels was rarely sustained and dose-limiting rashes occurred in some patients at 400mg. In an escalated schedule, 600mg seemed to give a sustained reduction in p24 levels for a few months (Cheeseman 1992). This might be the first indication of treatment efficacy, despite resistance development which was rapid both with nevirapine alone and in combination with zidovudine (Richman 1992).

## *7. Protease Inhibitors*

HIV protease has been intensively studied as a target for new anti-HIV drugs, but clinical data on efficacy are not yet available. It is a small dimeric





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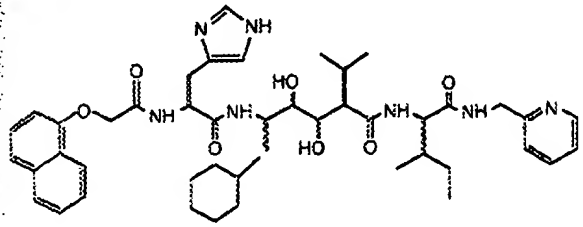


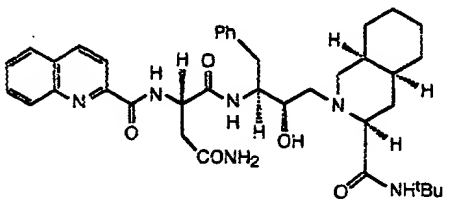


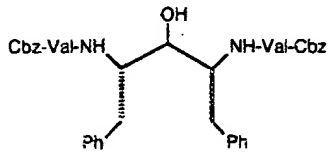


Fig. 5. Protease inhibitors being evaluated as potential drugs against HIV infections.

aspartic protease required for the specific cleavage of HIV polyprotein precursors both in virus particles during their maturation and in the infected cell (Debouck 1992; Huff 1991).

Very potent and selective *in vitro* inhibitors have been made, and their interactions with the protease studied by x-ray crystallography. Two hurdles were initially recognised with most inhibitors, namely low oral absorption and a short plasma $t_{1/2}$. These may now have been overcome, but a third possible problem is resistance development which has just begun to be reported, and its impact can not yet be fully assessed. Only a few compounds have reached clinical trials but many more are coming through. Most compounds are transition state mimics (fig. 5) of the normal substrates cleaved by HIV protease.

A phase I study of oral Ro-318959 at a dosage range of 25 to 1800mg 3 times daily in healthy subjects indicated that at 1800 mg/day oral bioavailability was 4% and the mean $t_{1/2}$ 12 hours. Phase I/II dose-finding trials in HIV-infected patients using oral Ro-318959 at 25 to 600mg 3 times daily and also in combination with zidovudine are under way.

A-80987, which is structurally similar to A-74704, has been reported to have an oral availability of 13 to 25% in animals and a terminal $t_{1/2}$ of 1.5 to 2 hours, and activity against HIV in cell cultures at 0.13 μmol/L (Norbeck et al. 1992). This compound was active against zidovudine-resistant HIV-1 and against HIV-2.

Clinical results for protease inhibitors are awaited with great interest and expected during 1993.

## *8. Other Inhibitors*

Several other inhibitors are in various stages of development but at present it is too early to identify any very promising compounds. Only a few will be mentioned here.

### 8.1 Glycosylation Inhibitors

This type of inhibitor has been reviewed by Ratner (1992). Several HIV proteins are heavily glycosylated, but the enzymes performing this are cellular and thus this function is likely to give toxicity problems. *N*-Butyl-deoxynojirimycin is currently in clinical trials. Diarrhoea has been seen but efficacy evaluation has not been reported.

Synergy with zidovudine, didanosine and zalcitabine has been observed with *N*-butyl-deoxynojirimycin and castanospermine, another inhibitor of glycosylation.

### 8.2 CD4

The CD4 glycoprotein serving as a receptor for HIV binding to T cells and monocytes has been evaluated as a potential drug against HIV infection and AIDS. Recombinant soluble CD4 (sCD4) lacking the CD4 transmembrane portion, CD4 coupled to immunoglobulin G, and CD4 coupled to toxins have also been made.

At almost the same time, several groups showed promising effects of sCD4 on HIV infection in cell cultures (Deen et al. 1988; Fischer et al. 1988; Hussey et al. 1988; Smith et al. 1987; Traunecker et al. 1988). However, in contrast to laboratory strains of HIV, primary HIV-1 isolates in high cell densities were less sensitive and high concentrations of sCD4 were required for neutralisation (Daar et al. 1990; Layne et al. 1991), making it difficult to reach dose levels necessary for therapeutic effects in patients.

A short $t_{1/}$ of sCD4 after subcutaneous administration (Kahn et al. 1990) has led to attempts to make recombinant human CD4-immunoglobulin G (rCD4-IgG). This conjugate has a prolonged *in vivo* terminal $t_{1/2}$ of about 2 days after intravenous administration (Hodges et al. 1991).

In clinical trials sCD4 (Kahn et al. 1990; Schooley et al. 1988, 1990) and rCD4-IgG (Hodges et al. 1991) have not given convincing results in patients with AIDS or ARC. Nevertheless, the treatments were well tolerated and the possibility of using rCD4-IgG in HIV-positive women during the last trimester of pregnancy is being studied in an attempt to prevent infection of newborns. Earlier studies in chimpanzees showed that intravenous administration of rCD4-IgG 5 mg/kg 8 hours and 1 hour before virus inoculation with cell-free virus protected the animals from infection (Ward et al. 1991).

The possible future clinical use of sCD4, rCD-IgG or CD4 coupled to toxins is uncertain.

### 8.3 Antisense RNA

Regulation of gene expression by antisense RNA (antimessenger RNA) occurs in eucaryotes (Gordon et al. 1988) and, in principle, an antisense RNA could regulate expression of any gene. HIV has been shown to be inhibited in cell cultures by endogenously synthesised antisense RNA (Rhodes & James 1990). Many types of antisense RNA with improved stability directed against conserved sequences have been synthesised. Such compounds can inhibit HIV replication when added to infected cells as first shown by Zamecnik et al. (1986). As tools to understand molecular events in cells, the synthetic antimessengers are of great interest but for clinical use against HIV or other diseases several hurdles such as cost of synthesis, pharmacokinetic problems (oral administration) and potency have to be overcome. Alternatives such as using viral vectors or ribozyme attached to the antisense RNA are still laboratory tools.

### 8.4 Inhibitors of Regulatory Proteins

There are several HIV regulatory proteins which may be used as targets (Rosen 1992), but Tat and Rev have been considered most attractive.

A Tat inhibitor, Ro-53335, has been described by Hsu et al. (1991). This compound is active against both HIV-1 and 2 in cell cultures at concentrations of 0.1 to 1 μmol/L and is also active against zidovudine-resistant virus. The rate of resistance development has not been reported.

Ro-53335 is in phase I/II clinical trials but results have not been published yet.

### 8.5 Hypericin

This compound was originally derived from the herb *Hypericum triquetrifolium* but has also been synthesised. The mechanism of action is not clear. A phase I dose-escalating trial has shown dose-limiting photosensitivity at intravenous doses of 0.5mg, elevated liver enzymes and a $t_{1/2}$ of about 24 hours. The potential use of hypericin cannot be predicted today.

## *9. Immunomodulators*

### 9.1 Inosine Pranobex

Inosine pranobex (isoprinosine), which nonspecifically enhances the mitogenic effect of various stimuli *in vitro*, has previously been reviewed (Sandström 1989). Two larger studies to evaluate efficacy have been performed, one in Denmark and Sweden and the other in England and the US. The results from the former showed a significant reduction in progression to AIDS among the recipients of inosine pranobex compared with placebo

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### 9.2 Ditiocarb Sodium

Early studies indicated that there could be some benefit of ditiocarb sodium (imuthiol, diethyldithiocarbamate) in patients with HIV disease, and indeed a meta-analysis was prompted. One placebo-controlled study of ditiocarb sodium in 389 symptomatic HIV-infected patients (± zidovudine) demonstrated a significant reduction in new opportunistic infections over a 24-week period (Hersch et al. 1991). A subsequent, as yet unpublished, large double-blind trial in asymptomatic patients that was terminated more than a year ago did not show any positive effect of ditiocarb sodium.

## *10. Active Immunisation*

There is an urgent need to develop alternative therapeutic interventions in HIV disease. Antiviral drugs such as zidovudine, didanosine or zalcitabine seem to be limited in that individuals with certain viral phenotypes may not respond at all and others may develop clinical or viral resistance within 0.5 to 2 years of therapy. Other approaches are needed for a more sustained effect alone or in combination with these drugs (Salk 1987).

HIV infection usually progresses over a prolonged period. The long time from infection to the development of complications and the slow decline of the immune system as indicated by the CD4+ count can be due to properties of HIV or the host response to the infection.

A number of observations indicate that the immune response plays a major role in controlling HIV infection, even though it is not successful in clearing the body of the virus and ultimately is defeated by the virus: (a) primary antigenaemia is cleared as antibodies develop in the acute stage of the disease; (b) both humoral and cell-mediated HIV-specific immunity are elicited; (c) there is an increased viral load as the immune system breaks down; (d) there is a rapid antigenic drift with selection of variants that differ from the original isolates, indicating some selective pressure; (e) there is selection of isolates that are resistant to neutralising antibodies directed against the V3 loop on the envelope protein; (f) active or passive immunisation has hindered infection by HIV-1 in chimpanzees; (g) chimpanzees experimentally infected with HIV develop a strong T cell response and do not progress to clinical disease.

The potential dangers of hyperimmunisation have so far not been borne out in studies of post-infection immunisation. The following cautions must however be borne in mind.

Increased immunological stimulation might activate HIV in general. In addition, those immune cells that are essential for control of HIV may be stimulated and make these cells susceptible to infection and destruction. In chimpanzees, a short burst of viral replication has been seen during the first week after immunisation.

Preliminary data from a trial with baculovirus produced by rgp160 failed to demonstrate any benefit in terms of reduced viral replication by zidovudine treatment in conjunction with immunisation (Bratt et al. 1992). However, a reduction in plasma viral RNA has been observed in several patients immunised with baculovirus-produced rgp160 after more than 1 year of therapy (Redfield, personal communication).

The rgp160 immunogen might attach to non-infected cells and mark them for destruction. This process could conceivably also induce autoimmune phenomena. The immunogen is however present locally in a complex with the adjuvant. So

far no evidence for these mechanisms has been documented.

Since low levels of antibodies that are neutralising in high concentrations might have an enhancing effect on HIV infectivity *in vitro*, there is a concern that a similar phenomenon operates *in vivo*.

A given vaccine might be inappropriate for immunisation of individuals infected by different strains. The most extensive experience today exists with baculovirus-produced rgp160 (Blick et al. 1992; Bratt et al. 1992; Redfield et al. 1991; Tsoukas et al. 1992; Valentine et al. 1992). This immunogen is derived from HIV-1 strain LAV/IIIB and is not 'typical' of the populations in the US, Europe or Africa. It might thus be argued that it is inappropriate for use in areas where the V3 loop of the immunogen does not correspond to that of the dominating strain populations, since the V3 loop appears to harbour the major epitopes for neutralising antibodies. On the other hand, it has been shown that the virus populations present at later times differ from the infecting isolates in their sensitivities to the initial neutralising anti-V3 antibodies. Furthermore, after immunising with strain IIIB, increased titres and affinities have been noted to heterologous viral strains, indicating anamnestic responses. Lastly, it might be desirable to hyperimmunise individuals to antigenic determinants that are normally nonimmunogenic, but play a role in controlling infection.

The baculovirus product differs from the naturally glucosylated product in that it is rich in mannose and lacks other sugar moieties. Other vaccine candidates, rgp120 and rpg160, are produced with vaccinia constructs in mammalian cells using IIIB or MN-like sequences (Birx et al. 1992). Sequences similar to isolate MN are much more prevalent than IIIB-like sequences in patients from North America and Western Europe. Placebo controlled double-blind trials are planned with these products as immunotherapy.

Envelope-depleted whole vaccines have been tried, and a correlation with specific delayed-type hypersensitivity and disease progression observed. A double-blind trial has just been terminated, but is not yet analysed (Turner et al. 1992).

The optimal vaccine dose has not been determined, even in terms of cell-mediated and humoral immunity (Birx et al. 1992; Redfield et al. 1991). It has nevertheless been found that 6 injections yield better antibody responses than 3, and that 160μg gives a better response than lower doses. However, it is not proven that the criteria for immunological response correlate with clinical efficacy.

Preliminary results from the 3 phase I trials in infected patients currently underway in the US, Canada and Sweden with baculovirus-produced rgp160 have not demonstrated any serious adverse effects. All studies have so far demonstrated a stabilisation or increase in CD4+ levels with 1 to 3 years of follow-up.

The decay of the immune response to immunisation with rgp160 is not well known but has been estimated to be in the order of 2 to 6 months. This has led to the practice of reimmunising patients every 2 to 4 months. It is claimed that little decrease in CD4+ counts is seen with this schedule (Redfield, personal communication). It is, however, unknown whether long term boosting is necessary to maintain the CD4+ levels or if this constant stimulation may have long term hazardous effects.

## 11. Passive Immunotherapy

Initial positive results in limited numbers of patients have been reviewed previously (Sandström 1989). Although little new information has emerged, interest has been rekindled by the ability to produce human or chimeric antibodies with synergistic properties against the V3 loop and CD4 binding site on gp120 (Pinter et al. 1992; Zolla-Pazner et al. 1992). With the surge of vaccine studies, there will soon be well-characterised immunoglobulin preparations available from uninfected vaccinated individuals available for clinical trials.

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## 12. Conclusions

Early introduction of zidovudine is clearly of benefit for a subgroup of patients. There is still doubt whether these observations extend to all patients, or whether there is a subgroup of patients with stable CD4+ counts and CD4+ function in whom antiretroviral therapy should be postponed.

It has been shown that progression to severe HIV disease is reduced by approximately 50% whether the CD4+ level in the study selects for patients with a risk of progression of 5% over 2 years or 10% over 1 year. Treatment has been well tolerated, but data are not yet available to show that the effect on CD4+ counts is more pronounced when treatment is introduced earlier. However, the rate of viral resistance appears to be slower. In patients with early HIV disease it is not clear if there is a benefit on survival, either in the short term (which has been studied) or in the long term (which probably can never be clarified).

In the Australian-European study with zidovudine, involving patients with CD4+ counts of 200 to 400 × $10^6$/L, the proportion of placebo-treated patients with CD4+ counts remaining above baseline levelled off at about 30% of those remaining in the study after 1 year. This could of course be because of selective withdrawal of patients with disease progression, but remains an important observation.

### 12.1 Duration of Zidovudine Monotherapy before Switching to Didanosine

Studies are emerging that demonstrate that there is some correlation between viral resistance and prognosis. Resistance in late HIV disease is found to develop in 6 to 12 months, but many patients have fully sensitive virus after 2 to 3 years. The phenotype of the virus, syncytia-inducing or non-syncytia-inducing, may be of much greater importance. It thus becomes illogical to specify a certain time, for example 1 year, when therapy should be changed.

This leads to the question of whether the resistance determination or viral phenotyping should be the decisive factors in altering treatment. Although these techniques may be available in certain settings, their prognostic importance still rests on very limited information and may currently in fact only complicate individual treatment decisions. Information on resistance development and removal of a drug that probably no longer inhibits the virus is however important to avoid unnecessary use of a drug with side effects. At present, clinical deterioration or declining CD4+ counts associated with disease progression seem to be the best factors for determining a change in therapy, irrespective of the length of zidovudine treatment and resistance data.

### 12.2 Should a New Drug be Added or Substituted after Failure of Zidovudine and Didanosine?

Data from one study indicate that the addition of a new drug could antagonise zidovudine inhibition in cells infected with zidovudine-resistant virus (Cox et al. 1992). Resistance mutations induced against one antiviral drug may induce sensitivity (or resistance) to another. In the absence of selective pressure, sensitive virus only slowly reappears, indicating that at least some mutants multiply almost as well as wild-type virus. This might mean that a change to an alternative drug is a first choice to try to speed up this process. If this is an effective process, subsequent alternating therapy could be considered. Alternatively, a combination therapy might be started once virus sensitive to zidovudine is re-established, so that resistance mutations to didanosine or TIBO-like compounds will prohibit the establishment of virus highly resistant to zidovudine.

If future observations confirm that virus growing well in MT-2 cells (i.e. syncytia-inducing phenotype) is not sensitive to zidovudine and possibly also other drugs, it may be unsuitable as monotherapy in patients with CD4+ counts below 400 × $10^6$/L, when these phenotypes start to appear. Whether zidovudine can be used at all or if combination treatments are advisable in patients with syncytia-inducing virus should be addressed

soon. Combination therapy for all may be a clinical choice if viral phenotyping is not available. So far there is limited experience with alternating therapies, with doses that are higher than those tolerated as monotherapy. The antiviral effect may well be as good with these high doses as the synergy of the combination, with the toxicity favouring the combination. The development of phenotypic and viral resistance may, however, be profoundly different.

### 12.3 New Drugs and Combinations Expected over the Coming Years

It seems that there will be further drugs which inhibit HIV reverse transcriptase. This might give possibilities to combine or alternate treatments where resistance to one inhibitor may increase the sensitivity to another. Such as yet only theoretical combinations or alternating regimens could then decrease problems of both toxicity and resistance development.

HIV protease inhibitors are probably becoming more useful agents. As with the reverse transcriptase inhibitors, combinations of protease inhibitors and combination with reverse transcriptase inhibitors to minimise toxicity and viral resistance are likely.

Combination regimens may also be useful to inhibit HIV replication in different types of cells *in vivo*. It is possible that the inhibitory potential, especially of nucleoside analogues, will depend on the type of cell treated, and many different cell types are infected in a patient.

The many new inhibitors and combinations already found to be active *in vitro* must be evaluated in appropriate *in vivo* models, probably by using monkeys infected with HIV-1, HIV-2 and SIV, to select suitable compounds, dosages and combinations for clinical investigation.

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Correspondence and reprints: Dr *Eric Sandström*, Södersjukhuset, Department of Dermatovenerology, S-118 83 Stockholm, Sweden.

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# Antibody Response to Viral Proteins U (*vpu*) and R (*vpr*) in HIV-1-Infected Individuals

†Peter Reiss, *†Joep M. A. Lange, *Anthony de Ronde, *Frank de Wolf, *John Dekker, †Sven A. Danner, ‡Christine Debouck, and *Jaap Goudsmit

*Departments of *Virology and †Internal Medicine (Academic Medical Centre), University of Amsterdam, Amsterdam, The Netherlands; and ‡Department of Molecular Genetics, Smith Kline and French Laboratories, King of Prussia, Pennsylvania*

**Summary:** Antibodies to *E. coli*-produced HIV-1 *vpr* and *vpu* were determined by enzyme immunoassay in serial sets of sera from 72 men seroconverting for antibodies to HIV-1 structural proteins, and from 196 initially symptom-free men who were positive for such antibodies at study entry. First detection of *vpr*- and *vpu*-specific antibodies always was within 12 months of seroconversion for antibodies to structural proteins. In the combined cohort of 268 men, *vpr*- and *vpu*-specific antibodies were found persistently in 26 and 43% of men, respectively. *Vpr*- and *vpu*-specific antibodies were transiently detected in 3 and 7%, respectively, and intermittently detected in 18 and 13% of men. *Vpr*- and *vpu*-specific antibodies were not detected in 53 and 37% of men, respectively. No association was found between the patterns of *vpr*- or *vpu*-specific antibody response and clinical outcome. In subjects with different patterns of *vpr*- and *vpu*-specific antibody response, no clear temporal relationship existed between the appearance or disappearance of antibodies and the onset of HIV-1-related disease. Key Words: *vpr*-specific antibodies—*vpu*-specific antibodies—HIV-1 infection.

Apart from the *gag*, *pol*, and *env* genes, coding for structural proteins, the genome of HIV-1 contains six known accessory genes (1), of which *tat*, *rev*, and *nef* code for proteins shown to have regulatory properties with respect to viral replication in vitro. Antibodies to all accessory gene-encoded proteins, including viral proteins U and R (*vpu* and *vpr*) can be found in sera from HIV-1-infected individuals (2–21). Whereas numerous reports concerning antibody response to *tat*, *rev*, and *nef* exist, including some showing associations with clinical outcome (6,7,20,21), information regarding the clinical significance of *vpr*- and *vpu*-specific antibodies in HIV-1-infected individuals is limited. One study (13) reported no clear association between the seroprevalence of antibodies to *vpr* and the clinical stage of infection with HIV-1. Another study (14) found a bimodal distribution of prevalence of antibody to *vpu*, with one peak associated with early clinical stages and another peak associated with the late stage of infection with HIV-1. The function of the *vpr* gene product remains unknown, but it does not seem to be required for viral replication (13,22). The *vpu* gene-encoded protein may have a role in virus assembly or maturation (17). Although a functional *vpu* gene is not absolutely essential for the production of virions, an HIV-1 mutant lacking a functional *vpu* gene demonstrated a lower rate of viral replication when compared to wild-type virus (17).

Address correspondence and reprint requests to Prof. Dr. J. Goudsmit at Human Retrovirus Laboratory L1-157, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands.

In the present study, patterns of antibody response to *vpr* and *vpu* were determined in serial sets of sera from 72 individuals seroconverting for antibodies to HIV-1 structural proteins and from 196 initially symptom-free subjects with known seropositivity for such antibodies. The incidence of HIV-1-related disease was compared between subjects with different *vpr*- and *vpu*-specific antibody responses. In a cross-sectional study, it was attempted to confirm the previously described bimodal distribution of *vpu*-specific antibodies, in subjects with different clinical stages of HIV-1 infection.

## METHODS

### Study Populations

Between October 1984 and March 1986, 961 asymptomatic men, living in and around Amsterdam and with at least two homosexual contacts in the preceding 6 months, were enrolled in a prospective study on the prevalence and incidence of HIV infection and risk factors for AIDS. Epidemiological and clinical data were collected, and blood was sampled every 3 months (23).

In the first serum sample taken, 723 men were found to be seronegative for HIV-1 antibody and 238 were found to be seropositive, using a commercially available enzyme immunoassay (EIA) based on purified human T-lymphotropic virus type IIIB (HTLV-IIIB) as antigen (Vironostika, Organon Teknika, Oss, The Netherlands). Seropositivity was confirmed by immunoblotting as previously described (24). During follow-up until April 1988, 76 of the men initially seronegative for HIV-1 antibody seroconverted, as shown by using a commercially available EIA based on *gag* and *env* encoded protein fragments (recombinant HTLV-III EIA, Abbott Laboratories, North Chicago, IL, U.S.A.). All available stored sequential serum samples from subjects who were seropositive or seroconverted for antibodies to HIV-1 structural proteins were tested retrospectively for *vpr*- and *vpu*-specific antibodies. In 42 of the seropositive men and 4 of the men who seroconverted, follow-up was too short and insufficient data on *vpr*- and *vpu*-specfic antibodies were obtained. The mean duration of follow-up for the remaining 196 men who were seropositive at entry was 33.3 months (range of 6–45 months) and for the remaining 72 men who seroconverted it was 20.8 months (range of 3–38 months).

During follow-up until April 1988, 34 of the seropositive men and 4 of the seroconverters developed AIDS [Centers for Disease Control (CDC) stages IVC1 or IVD]. In 5 of these 34 seropositive men, AIDS-related conditions (CDC IVA in 4 and autoimmune thrombocytopenia in 1) had been diagnosed 1–5 months prior to AIDS. In nine other seropositive men and two other seroconverters, AIDS-related conditions were diagnosed (CDC IVA in seven, persistent oral thrush in two, hairy leukoplakia in one, and HIV-related psoriasis in one).

Serum samples obtained between October 1985 and December 1988 from 29 HIV-1 antibody-seropositive homosexual men, at the time they had AIDS-related conditions (CDC IVA in 28, persistent oral thrush in 1) diagnosed in the AIDS clinic of the Academic Medical Centre, Amsterdam, The Netherlands, were also tested for *vpu*-specific antibodies. During follow-up until February 1989, AIDS was diagnosed in 14 of these men, 7 of whom were being treated with zidovudine. Of the remaining seven patients, who were not receiving zidovudine, serum samples from four were available for *vpu*-specific antibody detection at the time of diagnosis of AIDS.

Therefore, by combining the data from both study populations above, the prevalence of *vpu*-specific antibodies could be assessed in a total of 42 patients with AIDS and 45 patients with AIDS-related conditions. The prevalence of *vpu*-specific antibodies in symptom-free HIV-1-infected individuals was determined in the last available serum samples from the 219 men in the above longitudinally followed cohort, who had remained symptom-free at the end of follow-up.

### Detection of *vpu*- and *vpr*-Specific Antibodies

*vpr* and *vpu* were produced in *E. coli* as galactokinase fusion proteins. The bacterially synthesized *vpr* and *vpu* were purified to greater than 95% homogeneity and EIA was performed as described by Goudsmit et al. (25), except that horseradish peroxidase-labeled goat anti-human IgG (KPL, Gaithersburg, MD, U.S.A.) was used, instead of biotinylated goat anti-human IgG with streptavidin–biotinylated horseradish peroxidase complex. The last obtained serum samples of 100 of the longitudinally followed consistently HIV-1 antibody seronegative homosexual men were used as controls. A sample was considered to contain *vpr*- or *vpu*-specific antibodies if OD (sample) > OD (average of

100 HIV-1 antibody negative control samples) + 4SD.

### Classification of Patterns of *vpr*- and *vpu*-Specific Antibodies in Serial Serum Samples from Men Seroconverting or Seropositive for Antibodies to HIV-1 Structural Proteins

Five different patterns were distinguished: (a) *vpr*- or *vpu*-specific antibodies were detected in all samples (persistently positive). (b) No *vpr*- or *vpu*-specific antibodies were found at the time of seroconversion for antibodies to structural proteins or entry into the study, but they subsequently appeared and remained detectable throughout follow-up (seroconversion for anti-*vpr* or anti-*vpu*). (c) *vpr*- or *vpu*-specific antibodies could not be detected in any of the samples (persistently negative). (d) *vpr*- or *vpu*-specific antibodies were found at the time of seroconversion to structural proteins or at entry into the study, but subsequently disappeared and remained undetectable throughout follow-up (transiently positive). (e) One or more samples in which antibodies to *vpr* or *vpu* were detected were preceded and followed by samples in which no antibodies to *vpr* or *vpu* were detected (intermittently positive).

The duration of follow-up for subjects with the different patterns of *vpr*- and *vpu*-specific antibody response is shown in Table 1.

### Classification of HIV-1-Related Disease

Clinical assessment was made according to CDC criteria (26). Patients with AIDS belonged to CDC group IVC1 (28 patients) or group IVD (14 patients; 12 with Kaposi's sarcoma, 2 with malignant lymphoma). Patients with AIDS-related conditions belonged to CDC group IVA (39 patients), IVC2 (4 patients), and IVE (2 patients).

### Statistics

$\chi^2$ tests were used for comparing groups.

## RESULTS

### *vpr*- and *vpu*-Specific Antibody Response in HIV-1 Antibody Seroconverting Men

The appearance of *vpr*- and *vpu*-specific antibodies in relation to seroconversion for antibodies to HIV-1 structural proteins is shown in Table 2. During the period of 9 months prior to HIV-1 antibody seroconversion, *vpu*-specific antibodies were not detected in any of the subjects. During this period, *vpr*-specific antibodies were detected in two subjects: in one 6 months before HIV-1 antibody seroconversion, and remaining detectable in all samples during 27 months of follow-up; in the other subject, *vpr*-specific antibodies were detected in only one sample 3 months before HIV-1 antibody seroconversion, but in none of the subsequent samples during 15 months of follow-up. *vpr*- and *vpu*-specific antibodies usually appeared within 3 months after HIV-1 antibody seroconversion. In total, *vpr*-specific and *vpu*-specific antibodies were found, at any one time, in 49% (35/72) and 61% (44/72) of subjects, respectively.

TABLE 1. *Duration of follow-up in a cohort of 268 HIV-1 antibody seropositive homosexual men, according to pattern of* vpr- *and* vpu-*specific antibody response*

| Pattern of antibody response | Anti-*vpr* | | Anti-*vpu* | |
|---|---|---|---|---|
| | HIV-1 Ab seroconverters | HIV-1 Ab seropositives | HIV-1 Ab seroconverters | HIV-1 Ab seropositives |
| Persistently positive (group 1) | 15 (6–30) | 30 (6–42) | 16 (3–38) | 32 (10–42) |
| Seroconverting for *vpr* and *vpu* (group 2) | 19 (5–33) | 30 (15–39) | 21 (14–30) | 33 (27–42) |
| Persistently negative (group 3) | 19 (3–36) | 32 (9–42) | 19 (3–36) | 30 (6–42) |
| Transiently positive (group 4) | 28 (24–31) | 31 (18–40) | 23 (9–30) | 36 (21–42) |
| Intermittently positive (group 5) | 24 (15–38) | 33 (11–45) | 26 (10–33) | 36 (20–45) |

Mean number of months followed-up. Numbers in parentheses are the ranges in the months followed-up.

TABLE 2. *Appearance of antibodies to* vpu *and* vpr *in 72 HIV-1* gag/env *antibody seroconverting homosexual men*

| | Time (months) relative to HIV-1 *gag/env* antibody seroconversion | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | −9 | −6 | −3 | 0 | 3 | 6 | 9 | 12 | 15 | 18 | 21 | 24 |
| Anti-*vpu* | 0/4 (0%) | 0/57 (0%) | 0/68 (0%) | 26/72 (36%) | 34/70 (49%) | 23/64 (36%) | 17/60 (28%) | 18/55 (33%) | 16/51 (31%) | 16/46 (35%) | 12/40 (30%) | 10/32 (31%) |
| Anti-*vpr* | 0/4 (0%) | 1/57 (2%) | 2/68 (3%) | 10/72 (14%) | 25/70 (36%) | 21/64 (33%) | 23/60 (38%) | 22/55 (40%) | 14/51 (27%) | 10/46 (22%) | 11/40 (28%) | 8/32 (25%) |

## Patterns of *vpr*- and *vpu*-Specific Antibody Response

The prevalence of the different patterns of *vpr*- and *vpu*-specific antibody response in the longitudinally studied seroconverting and at entry HIV-1 antibody seropositive men is shown in Table 3. In 126/268 (47%) men, *vpr*-specific antibodies were detected. Detection was persistent from the moment of HIV-1 antibody seroconversion or entry into the study in 51/268 (19%) men, and following seroconversion for *vpr*-specific antibodies in 18/268 (7%) men. Detection of *vpr*-specific antibodies was transient in 11/268 (4%) and intermittent in 46/268 (17%) of men. No *vpr*-specific antibodies were detected in 142/268 (53%) of men. In a small majority of subjects, *vpu*-specific antibodies were found (169/268 men; 63%). In 101/268 (38%) of subjects, *vpu*-specific antibodies were persistently detectable from the moment of HIV-1 antibody seroconversion or entry into the study, and in 14/268 (5%) following seroconversion for *vpu*-specific antibodies. In 20/268 (7%) of subjects, *vpu*-specific antibodies were transiently and in 34/268 (13%) intermittently detectable. In 99/268 (37%) of subjects, no *vpu*-specific antibodies were detected.

Among the HIV-1 antibody seroconverting men, a higher percentage of men seroconverted for anti-*vpr* and anti-*vpu*, and among the men who were HIV-1 antibody seropositive at entry, a higher percentage was persistently positive for anti-*vpr* and anti-*vpu*. This difference could be expected since seroconversion for anti-*vpr* and anti-*vpu* always occurred within 12 months of seroconversion for antibodies to HIV-1 structural proteins. Both in the HIV-1 antibody seroconverting men and in the at entry HIV-1 antibody seropositive men, the levels of *vpr*-specific antibodies in the subjects belonging to groups 1 and 2 (Table 3) (mean OD ± SEM: 1,137 ± 86 and 1,100 ± 29 in group 1, 962 ± 87 and 931 ± 75 in group 2) appeared to be higher than in subjects belonging to groups 3, 4, and 5 (mean OD ± SEM: 238 ± 4 and 224 ± 2 in group 3, 817 ± 68 and 513 ± 18 in group 4, 518 ± 17 and 532 ± 10 in group 5). Similar results were obtained for the levels of *vpu*-specific antibodies (mean OD ± SEM: 1,343 ± 63 and 1,429 ± 21 in group 1, 1,253 ± 83 and 707 ± 36 in group 2 vs. 168 ± 4 and 198 ± 2 in group 3, 738 ± 66 and 470 ± 18 in group 4, 537 ± 37 and 512 ± 14 in group 5). We therefore decided, for analysis of the relationship between clinical outcome and the patterns of *vpr*- and *vpu*-specific antibodies, to

TABLE 3. *Antibody response to* vpr *and* vpu *in a cohort of 268 HIV-1 antibody seropositive homosexual men*

| Pattern of antibody response | Anti-*vpr* | | | Anti-*vpu* | | |
|---|---|---|---|---|---|---|
| | HIV-1 Ab seroconverters, no. (%) | HIV-1 Ab seropositives, no. (%) | Total, no. (%) | HIV-1 Ab seroconverters, no. (%) | HIV-1 Ab seropositives, no. (%) | Total, no. (%) |
| Persistently positive (group 1) | 9 (12) | 42 (21) | 51 (19) | 16 (22) | 85 (43) | 101 (38) |
| Seroconverting for *vpr* and *vpu* (group 2) | 10 (14) | 8 (4) | 18 (7) | 7 (10) | 7 (4) | 14 (5) |
| Persistently negative (group 3) | 40 (56) | 102 (52) | 142 (53) | 32 (45) | 67 (34) | 99 (37) |
| Transiently positive (group 4) | 2 (3) | 9 (5) | 11 (4) | 11 (15) | 9 (5) | 20 (7) |
| Intermittently positive (group 5) | 11 (15) | 35 (18) | 46 (17) | 6 (8) | 28 (14) | 34 (13) |

gather subjects from groups 1 and 2 (anti-*vpr* and anti-*vpu* persistently positive and seroconverting for anti-*vpr* and anti-*vpu*, respectively), on the one hand, and from groups 3, 4, and 5 (anti-*vpr* and anti-*vpu* persistently negative, transiently positive, and intermittently positive, respectively) on the other hand. This also permitted us, for the purpose of this analysis, to consider both the HIV-1 antibody seroconverting and at entry seropositive men as a whole.

The groups were analyzed separately for the temporal relationship between *vpr*-specific and *vpu*-specific antibody response, and the onset of HIV-1-related disease.

### Association Between *vpr*- and *vpu*-Specific Antibody Response and Clinical Outcome of Infection with HIV-1

No association was found between patterns of *vpr*- or *vpu*-specific antibody response and clinical outcome ($p = 0.59$ and $p = 0.29$) (Tables 4 and 5). When all subjects with detectable *vpr*- or *vpu*-specific antibodies were gathered (groups 1, 2, 4, and 5 in Table 3) and compared with subjects without detectable *vpr*- or *vpu*-specific antibodies (group 3 in Table 3), again no associations with clinical outcome were found (data not shown).

The temporal relationship between the presence or appearance and absence or disappearance of *vpr*- and *vpu*-specific antibodies and the onset of AIDS or AIDS-related conditions is shown in Fig. 1.

Twelve of the 51 (24%) individuals with persistently detectable *vpr*-specific antibodies developed AIDS ($n = 10$) or AIDS-related conditions ($n = 2$). One of these men was a known HIV-1 antibody seroconverter and developed persistent oral thrush 12 months after seroconversion. Two of the 18 (11%) subjects who seroconverted for *vpr*-specific antibodies developed AIDS. In one of these men, *vpr*-specific antibodies appeared 3 months after HIV-1 antibody seroconversion and Kaposi's sarcoma was diagnosed 21 months thereafter. Twenty-three of the 142 (16%) subjects who were persistently negative for *vpr*-specific antibodies developed AIDS ($n = 18$) or AIDS-related conditions ($n = 5$). Three of these were known HIV-1 antibody seroconverters, and developed AIDS 11, 18, and 30 months after seroconversion. Three of the 11 (27%) subjects who were transiently positive for *vpr*-specific antibodies, all HIV-1 antibody seropositive at entry, developed AIDS ($n = 1$) or AIDS-related conditions ($n = 2$). Nine of the 46 (20%) subjects who were intermittently positive for *vpr*-specific antibodies developed AIDS ($n = 7$) or AIDS-related conditions ($n = 2$). One of these men developed CDC IVA 18 months after HIV-1 antibody seroconversion, which was 18 months after first and 9 months after last detection of *vpr*-specific antibodies.

Twenty of the 101 (20%) men with persistently detectable *vpu*-specific antibodies developed AIDS ($n = 13$) or AIDS-related conditions ($n = 7$). One of these men was a known HIV-1 antibody seroconverter and developed persistent oral thrush 12 months after seroconversion. One of the 14 (7%) subjects who seroconverted for *vpu*-specific antibodies 6 months after HIV-1 antibody seroconversion developed Kaposi's sarcoma 12 months thereafter. Twenty of the 99 (20%) subjects who were persistently negative for *vpu*-specific antibodies developed AIDS ($n = 18$) or AIDS-related conditions ($n = 2$). Four of these were known HIV-1 antibody seroconverters, and developed AIDS after 11, 24, and 30 months and CDC IVA after 18 months. Two

TABLE 4. *Relationship of vpr-specific antibody response to clinical outcome of infection in 268 homosexual men who were positive for antibody to structural proteins of HIV-1*

| | No. of subjects (%) | | | |
|---|---|---|---|---|
| Anti-*vpr* response | CDC II/III | CDC IVA/IVC2/IVE | CDC IVC1/IVD | Total |
| Persistently positive/ seroconverting for anti-*vpr* (group 1/2) | 55 (80) | 2 (3) | 12 (17) | 69 |
| Persistently negative/ transiently positive/ intermittently positive (group 3/4/5) | 164 (82) | 9 (5) | 26 (13) | 199 |

CDC, Centers for Disease Control.

TABLE 5. *Relationship of vpu-specific antibody response to clinical outcome of infection in 268 homosexual men who were positive for antibody to structural proteins of HIV-1*

| Anti-*vpu* response | No. of subjects (%) | | | |
|---|---|---|---|---|
| | CDC II/III | CDC IVA/IVC2/IVE | CDC IVC1/IVD | Total |
| Persistently positive/ seroconverting for anti-*vpu* (group 1/2) | 94 (82) | 7 (6) | 14 (12) | 115 |
| Persistently negative/ transiently positive/ intermittently positive (group 3/4/5) | 125 (82) | 4 (3) | 24 (15) | 153 |

CDC, Centers for Disease Control.

of the 20 (10%) men who had transiently detectable *vpu*-specific antibodies, both HIV-1 antibody seropositive at entry, developed AIDS. Six of the 34 (18%) subjects with intermittently detectable *vpu*-specific antibodies, all HIV-1 antibody seropositive at entry, developed AIDS ($n = 4$) or AIDS-related conditions ($n = 2$).

### Prevalence of *vpu*-Specific Antibodies During Different Clinical Stages of HIV-1 Infection

Studied cross-sectionally in single serum samples, there was no significant ($p > 0.1$) difference in prevalence of *vpu*-specific antibodies between asymptomatic (CDC II/III) HIV-1 antibody seropositive men, and men with both early (CDC IVA/IVC2/IVE) and late (CDC IVC1/IVD) stages of HIV-1 related disease. These results are depicted in Fig. 2.

## DISCUSSION

We previously reported (10,27) the antigenicity of *E. coli*-produced *nef*, *rev*, and *tat* in the same cohort of HIV-1 antibody seroconverting men described in the present study. *nef* was found to be highly antigenic with *nef*-specific antibodies being detectable in 83% of individuals. *rev*- and *tat*-specific antibodies were clearly less prevalent, and found in 47 and 29% of men, respectively. The present data show *vpr*- and *vpu*-specific antibodies also to be less prevalent than *nef*-specific antibodies. At any one time, *vpr*-specific antibodies were found in 49% and *vpu*-specific antibodies in 61% of HIV-1 antibody seroconverting men. Very similar percentages of antibody responders were found when analyzing the groups of HIV-1 antibody seroconverting and at entry seropositive men as a whole. Like antibodies to *nef*, *rev*, and *tat*, *vpr*- and *vpu*-specific antibodies appeared early, i.e., at or within 12 months after seroconversion for antibodies to structural proteins. Several explanations can be offered for the lower prevalence of *vpr*- and *vpu*-specific antibodies compared to that of *nef*-specific antibodies, early after infection. First and most likely, the antigenicity of *vpr* and *vpu* may be lower than that of *nef*. Second, *vpr* and *vpu* are expressed early, but exposed to the immune system in only some individuals. Third, some individuals are not capable of mounting an antibody response to *vpr* and *vpu*, while others are. Fourth, *vpr* and *vpu* epitopes may be variable. Finally, antibodies to *vpr* and *vpu* may not be detectable in some individuals due to immune complex formation.

As reported previously (6,10) for *nef*-, *rev*-, and *tat*-specific antibodies, in sequential serum samples *vpr*- and *vpu*-specific antibodies could be persistently detectable (being present from the time of HIV-1 antibody seroconversion or entry into the study or appearing soon thereafter), transiently or intermittently detectable, or not detectable at all, respectively. No association was found between these patterns of *vpr*- and *vpu*-specific antibody response and the development of HIV-1-related disease. Also, no obvious temporal relationship existed between the appearance or disappearance of *vpr*- and *vpu*-specific antibodies and disease progression, although numbers were too small for definite conclusions.

In a cross-sectional study using single serum samples, Matsuda et al. (14), classifying subjects according to the Walter Reed staging system (28), found a higher prevalence of *vpu*-specific antibodies in patients with early (WR2 and WR3) and late (WR6) stages of infection, compared to patients with intermittent (WR4 and WR5) stages. It is un-

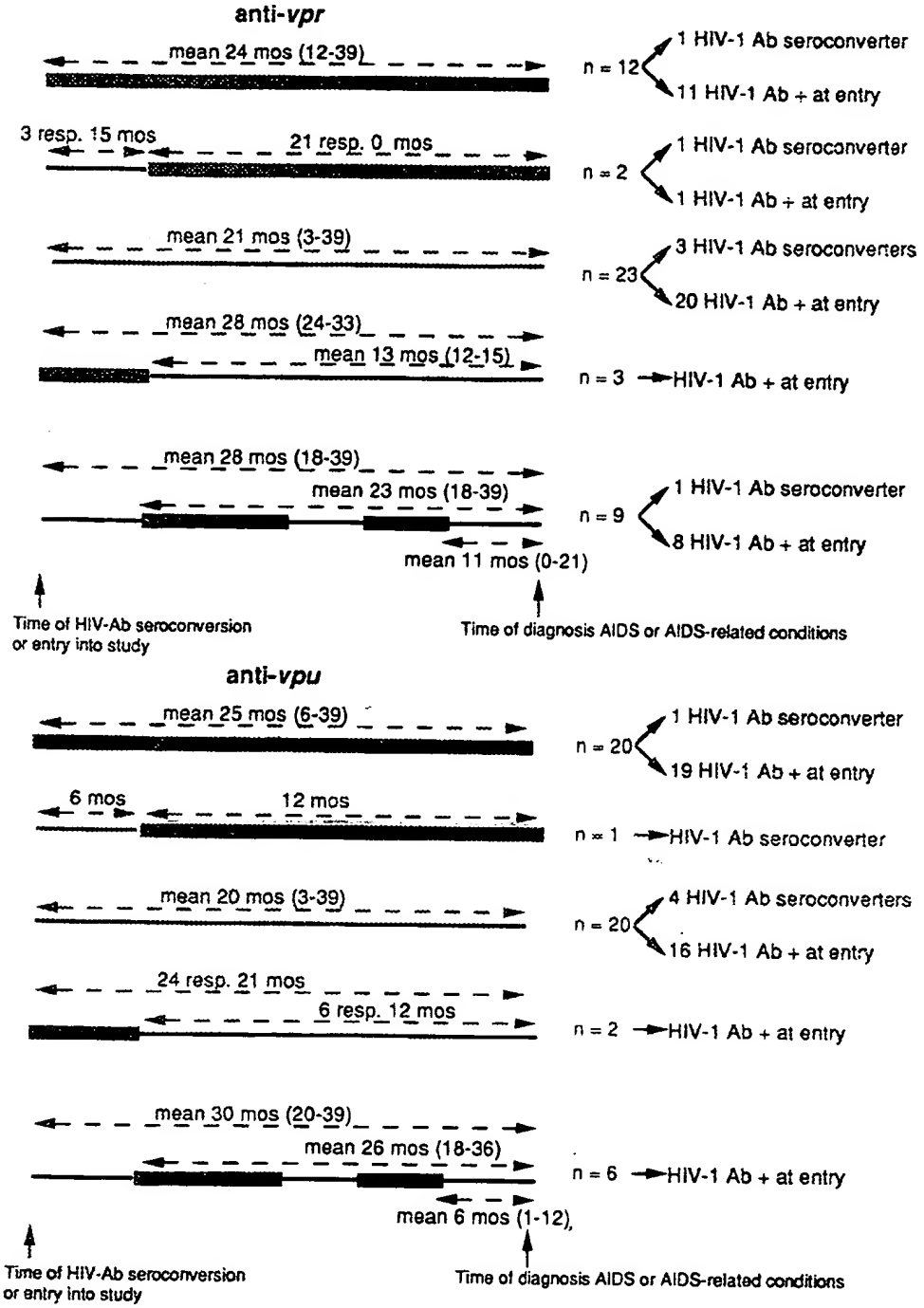


FIG. 1. Temporal relationship between patterns of *vpr*- and *vpu*-specific antibody response and onset of AIDS or AIDS-related conditions. Thick black bar, OD values above cut-off; thin black bar, OD values below cut-off.

clear, however, whether these differences were significant. We found no significant differences in prevalence of *vpu*-specific antibodies between groups of subjects with early, intermittent, and late stages of infection according to CDC criteria. One possible explanation for this discrepant result may be the difference in clinical staging system, but without more data concerning the clinical and demographic characteristics of the population studied by Matsuda et al., this can not be confirmed.

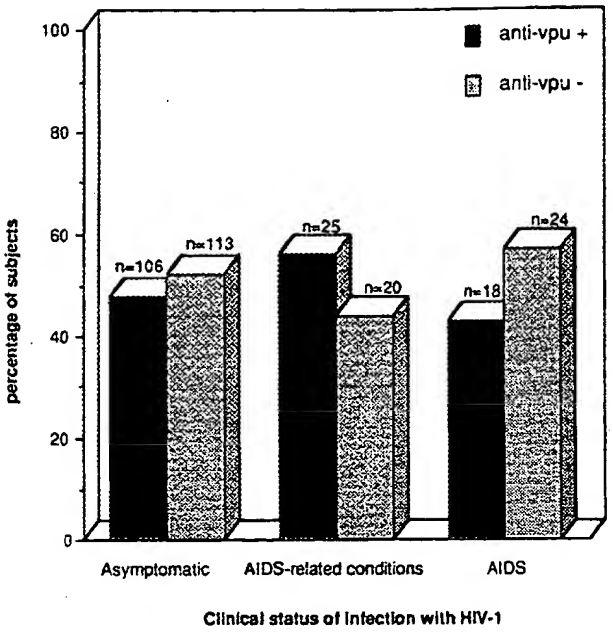


**FIG. 2.** Prevalence of *vpu*-specific antibodies in HIV-1 antibody seropositive men with asymptomatic infection (CDC II/III), AIDS-related conditions (CDC IVA/IVC2/IVE), and AIDS (CDC IVC1/IVD).

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# Identification and Localization of *vpr* Gene Product of Human Immunodeficiency Virus Type 1

AKIHIKO SATO,[1] HISANAGA IGARASHI,[1] AKIO ADACHI,[2] AND MASANORI HAYAMI[2]
[1]*Shionogi Institute for Medical Science, Settsu-shi, Osaka*
[2]*Institute for Virus Research, Kyoto University, Kyoto, Japan*

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Requests for reprints should be addressed to Akihiko Sato, Shionogi Institute for Medical Science, 2-5-1 Mishima, Settsu-shi, Osaka 566, Japan.

Key words: AIDS, HIV-1, *vpr*, identification, localization, Western

***Abstract***

The entire *vpr* gene of human immunodeficiency virus type 1 (HIV-1) was cloned into procaryotic and eucaryotic expression vectors. Production of authentic protein encoded by the gene in bacterial and mammalian cells was monitored by Western blotting using guinea pig antisera raised against an N-terminal 14-oligopeptide of the predicted *vpr* protein. A specific 12-kD protein was clearly detected with these antisera, but not with preimmune sera, in both cell systems, and this binding was blocked by the oligopeptide. These antisera also recognized a protein of the same size in several human T-cell lines infected with HIV-1. Western blotting analysis of subcellular fractions prepared from the cells producing wild-type *vpr* protein strongly suggested that the protein was membrane associated. A region within the *vpr* required for the stable expression of *vpr* product was also suggested by mutational analyses.

## Introduction

The genome of human immunodeficiency virus type 1 (HIV-1), a causative agent of acquired immunodeficiency syndromes, contains many extra open reading frames (OFRs) not found in other retroviruses (1–3). These include genes designated *vif*, *vpr*, *tat*, *rev*, *vpu*, and *nef* (4). Biochemical and genetic studies on the unique genes have revealed their products and functional roles in the replicative

cycle of the virus (5). However, little is known about the nature of *vpr*. The *vpr* ORF is well conserved, not only among primate lentiviruses, but also in the distantly related ungulate lentivirus, visna virus (6). This fact suggests that *vpr* plays an important role in the life cycle of the lentivirus group.

Recently, Wong-Staal et al. found that some infected individuals, but not uninfected individuals, possess antibodies reactive with a bacterially expressed HIV-1 *vpr* gene product (7). Ogawa et al. showed, by using proviral mutants of HIV-1 generated in vitro, that the *vpr* may encode a positive factor for virus growth, and also that the C-terminal portion of *vpr* product may be important for the functionality (8). Cohen et al. reported that the 15-kD *vpr* gene product is a transactivator of the HIV long terminal repeat (LTR) as well as the LTRs of other promoters (9). The latter two reports are consistent in that HIV-1 *vpr* positively regulates the replication of virus.

In order to better understand the *vpr* gene, we have cloned the *vpr* derived from a functional molecular clone pNL432 that expresses all known HIV-1 genes (10) into expression vectors and examined the products in the cells, along with the samples prepared from virus-infected cells, for the identification and localization of HIV-1 *vpr* protein. We demonstrate here that the HIV-1 *vpr* encodes a 12-kD protein that is associated with cellular membranes. In addition, we show that the removal of 13 amino acids in *vpr* abolished the stable expression of the *vpr* gene product.

## Materials and Methods

### *Cells*

$CD4^+$ human leukemia cell lines, A3.01 (8), Molt4 clone 8 (8), MT-2 (11), and MT-4 (11) were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. A human colon carcinoma cell line, SW480 (ATCC CCL-228), and a hamster kidney cell line, BHK21 (ATCC CCL-10), were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum.

### *DNA constructs*

The DNA constructs, a procaryotic expression vector pUC118N (12), a eucaryotic expression vector (pMAM-neo) (13), and an infectious proviral DNA clone of HIV-1 (pNL432) (10) have been described. Plasmid DNA pUC118N-R carrying the entire *vpr* gene of pNL432 was constructed as follows. A 3.2-kb *PstI-SacI* fragment containing the *vpr* gene was cloned into pUC118 and a *NcoI* site (5′-



A.

pNL432 vpr

MEQAPE

GDTWAG

B.

pUC118

pUC118

C.

pMAM-n

pMAM-n

*Fig. 1.* Predicted amino a
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the *vpr* gene. For details,
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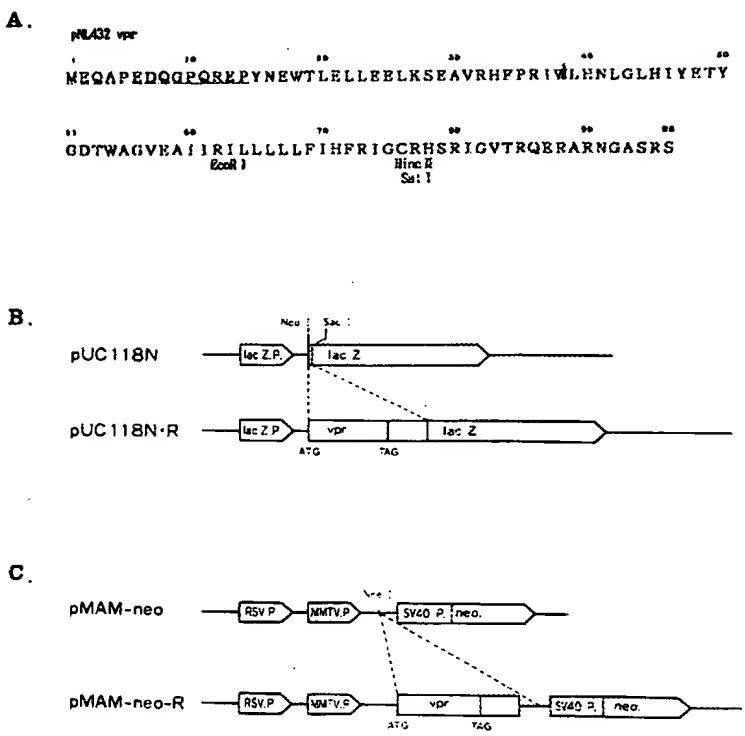


*Fig. 1.* Predicted amino acid sequence of HIV-1 *vpr* (432 strain), and of procaryotic and eucaryotic expression vectors carrying the *vpr* gene. *A:* Amino acid sequence of *vpr* deduced from the nucleotide sequence (8). The peptide synthesized to immunize guinea pigs is underlined. The restriction sites used to make mutants are also shown. *B,C:* Schematic representation of plasmid DNAs containing the *vpr* gene. For details, see Materials and Methods. P = promoter; RSV = Rous sarcoma virus; MMTV = mouse mammary tumor virus; SV40 = simian virus 40; neo = neomycin.

CCATGG-3′) was generated at the initiation codon (5′-AGATGG-3′) of *vpr* by oligonucleotide-directed in-vitro mutagenesis. The *Nco*I-*Sac*I fragment of this clone was put into pUC118N to obtain pUC118N-R (Fig. 1B). From this clone, pREN and pRHN were generated by insertion of a termination linker (5′-TAACTAGATAGC-3′) into the *Eco*RI or *Hin*cII sites, respectively. To construct pMAM-neo-R, another *Xba*I site was introduced into pUC118N-R by using *Sph*I-*Nco*I linker (5′-CATGAGCATGCC-3′) and *Xba*I linker (5′-CTCTAGAG-3′) (into the *Sph*I site created earlier). The resultant *Xba*I-*Xba*I fragment was ligated to the *Nhe*I site of pMAM-neo in a sense orientation to obtain pMAM-neo-R (Fig. 1C). Proviral mutants of *vpr* gene, designated pNLEN and pNLSN, were constructed by insertion of termination linker (as above) into the *Eco*RI or *Sal*I site respectively, of pNL432.

*Expression of vpr gene product*

To monitor the expression of *vpr* gene in bacteria, *Escherichia coli* JM109 cells were transformed with pUC118N, pUC118N-R, pREN, or pRHN, and stimulated by isopropyl-β-D-thiogalactopyranosid (IPTG). Transfection of pMAM-neo, pMAM-neo-R, pNL432, pNLEN, and pNLSN into the mammalian cells was carried out by the calcium phosphate coprecipitation method (14). To obtain BHK21 cells that constitutively express the *vpr* gene product, pMAM-neo (as a negative control) or pMAM-neo-R was introduced into the cells and geneticin (G418)-resistant cells were selected (15).

*Western blot analysis*

For Western blot analysis, bacterial cells were lysed in Laemmli's sample buffer (16) and mammalian cells were solubilized in 1% NP40-10mM Tris-HCl (pH 8.0)-1mM EDTA. Subcellular components, prepared as described previously (17), were solubilized in 8 M urea-1 M NaCl. Cell lysates (the same amount of protein was used) were resolved by 16% (Figs. 3, 4, and 6) or 18% (Figs. 2 and 5) sodium dodecyl sulfate-polyacrylamide gel electrophoresis ($NaDodSO_4$-PAGE) (16) and then electrophoretically transferred to nitrocellulose membranes. The membranes were incubated with anti-*vpr* guinea-pig sera raised against a synthetic N-terminal 14-oligopeptide of the predicted *vpr* protein (Fig. 1) and processed with the protein A-horseradish peroxidase (Zymed Laboratories, San Francisco, CA) and Konica immunostaining HRP kit (Konica & Co., Tokyo).

## Results

*Expression of vpr gene product in bacterial and mammalian cells*

There has been only one report concerning the direct identification of the HIV-1 *vpr* protein (9). To confirm and extend the data on structural analysis presented, we monitored the expression of *vpr* gene in two systems.

First, a series of procaryotic expression vectors carrying wild-type (wt) or mutated *vpr* gene were constructed. The wt gene came from an infectious clone that was reported to have biologically active *vpr* (8) and to encode 96 amino acids (Fig. 1A). All the plamid DNAs were designed to express the non-fusion-type *vpr* gene product (Fig. 1B; for details, see Materials and Methods). Thus, pUC118N-R (wt), pREN (mutant), and pRHN (mutant) encoded native 96, 63, and 76 (plus three irrelevant), respectively, amino acids from the N terminus of *vpr* protein (Fig. 1A). Fig. 2 shows the products synthesized in transformed JM109 cells as examined by Western blotting using anti-*vpr* peptide sera. pUC118N-R directed the synthesis of large amounts of 12-kD protein (lane 4), whereas no protein of



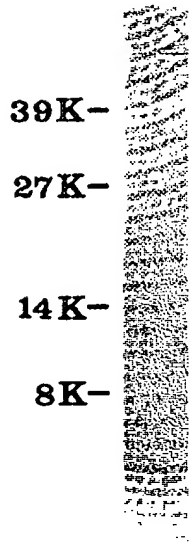


*Fig. 2.* Identification of *vpr* in the presence of 2 mM IP (lane 1), pREN (lanes 2 an was analyzed by Western b sorbed with the *vpr* peptid

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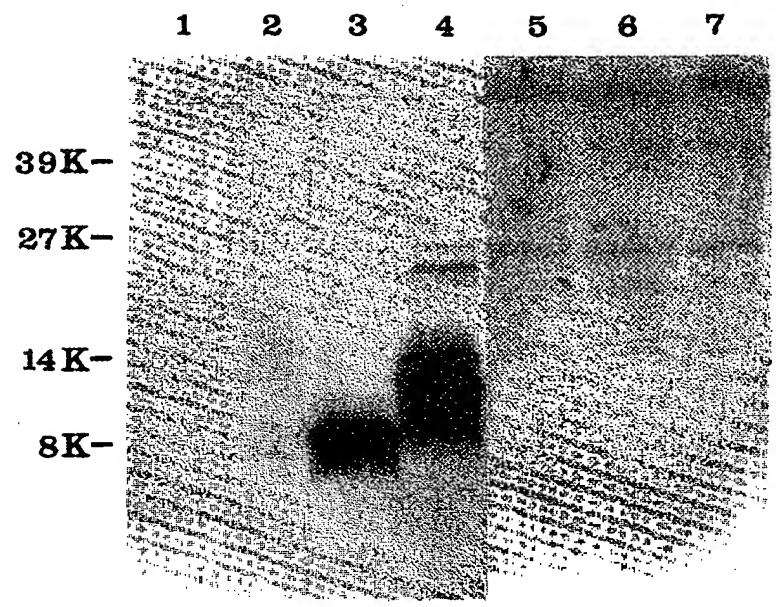


*Fig. 2.* Identification of *vpr* gene product in bacteria JM109 cells. JM109 transformants were cultured in the presence of 2 mM IPTG. Cell lysates were prepared from the cells transformed with pUC118N (lane 1), pREN (lanes 2 and 5), pRHN (lanes 3 and 6), or pUC118N-R (lanes 4 and 7). Each lysate was analyzed by Western blotting with either anti-*vpr* peptide sera (lanes 1–4) or anti-*vpr* sera preadsorbed with the *vpr* peptide (lanes 5–7).

a similar size that was reactive with the antisera was detected in the pUC118N-transformant (lane 1). Large amounts of the 9-kD (lane 3) and an extremely low level of 7-kD (lane 2) proteins were synthesized in pRHN-transformant and pREN-transformant, respectively. The specificity of reactivity with antisera was verified by completely blocking the reaction by the synthetic peptide used for immunization (lanes 5–7). The molecular mass of these proteins agreed well with the predicted size. The 14-kD band in lane 4 could be a read-through product (108 amino acids) due to the supresser gene SupE44 in JM109 cells (18). Another minor specific protein observed in lane 4 probably represented a dimer of the 12-kD protein because of its molecular size (24-kD).

Second, a stable BHK21 cell line that expressed the *vpr* gene was established. The construct pMAM-neo-R, which we used as a eucaryotic expression vector, contained *vpr* gene under the control of a dexamethasone-inducible promotor (Fig. 1C). Fig. 3 indicates the specific expression of the 12-kD protein in pMAM-neo-R-transformed BHK21 cells (lanes 3 and 4). As clearly shown, dexamethasone augmented several fold the synthesis of the 12-kD protein, and the bands disappeared by pretreating the antisera with the peptide (lanes 5 and 6). No reactivity with anti-*vpr* sera was seen in the cells transformed with the parental expression vector pMAM-neo (lanes 1 and 2).

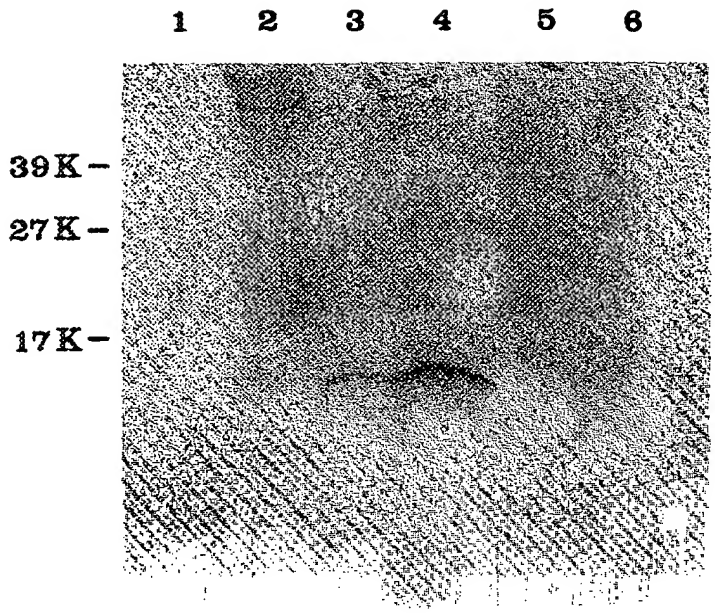


*Fig. 3.* Identification of the *vpr* gene product in BHK21 cells. G418-resistant BHK 21 cells were cultured in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of 2 μM dexamethazone. Cell lysates were prepared from pMAM-neo transformants (lanes 1 and 2) or pMAM-neo-R transformants (lanes 3–6). Each sample was analyzed by Western blotting with either anti-*vpr* sera (lanes 1–4) or anti-*vpr* sera preadsorbed with the *vpr* peptide (lanes 5 and 6).


*Fig. 4.* Localization of th
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(S100; lanes 3 and 4), or n
with anti-*vpr* peptide sera.

On the basis of our observation that the protein made by the *vpr* gene was of 12-kD molecular mass, we next tried to determine its subcellular localization in the *vpr*-producing BHK21 cells. The cells transformed with pMAM-neo-R were fractionated into cytosol, membrane, and nuclear components, and lysates prepared from each fraction were analyzed by Western blotting (Fig. 4). The *vpr* gene product was a 12-kD protein and was mainly detected in the lysates from membrane fraction (lane 5). The 12-kD protein was barely detectable in the lysates from two other fractions (lanes 1 and 3) and was not detectable at all in the controls (lanes 2, 4, and 6). Immunofluorescence and immunoperoxidase staining of BHK21 cells producing *vpr* protein confirmed the results described above.

*Expression of vpr protein in human CD4⁺ cells infected with HIV-1*

To determine whether the 12-kD protein is actually expressed and localized in the membrane fraction in the natural system, infected-cell lysates were monitored for the synthesis of *vpr* protein. As a control, two proviral *vpr* mutants, designated pNLEN and pNLSN, were constructed. These mutants encoded native 63

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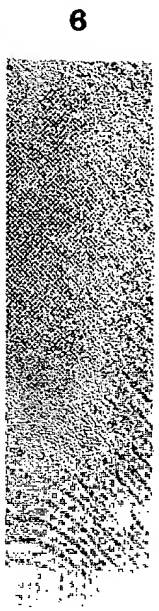


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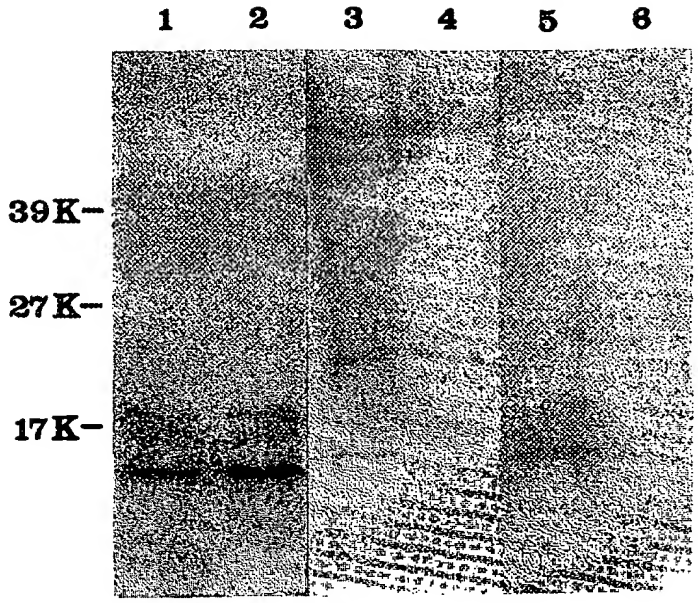


*Fig. 4.* Localization of the *vpr* gene product in BHK21 cells. pMAM-neo-R transformants (lanes 1, 3, and 5) and pMAM-neo transformants (lanes 2, 4, and 6) were fractionated into subcellular components and lysates were prepared. Lysates from the nuclear fraction (lanes 1 and 2), cytosol fraction (S100; lanes 3 and 4), or membrane fraction (P100; lanes 5 and 6) were analyzed by Western blotting with anti-*vpr* peptide sera.

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(pNLEN) and 77 (pNLSN) amino acids from the N terminus of *vpr* protein (Fig. 1A) and were expected to be infectious (8,9).

Human $CD4^+$ MT-2 cells were infected with viruses recovered from transfected SW480 cells (10) and checked for the expression of *vpr* and for virus growth. Both mutant viruses grew equally well with wt virus in MT-2 cells, and infected cells exhibited severe cytopathic effects typical to HIV. Kinetic studies of *vpr* expression as monitored by Western blotting revealed that the 12-kD protein was most easily detectable at the peak of virus production. The representative result is presented in Fig. 5. The 12-kD protein was readily detected in the lysates prepared from cells infected with wt virus (lane 2), whereas the 9-kD protein, the predicted size from the mutation, was synthesized in the cells infected with the mutant virus (pNLSN; lane 3). The specificity of the reaction was further confirmed by the negative effect of synthetic *vpr* peptide (lanes 4 and 5). Quite interestingly, viruses derived from pNLEN could not produce protein reactive with antisera (lane 1). These results were repeated in other experiments using MT4, Molt4 clone 8, and A3.01 cells as the target for infection, consistent with the data obtained with the bacterial system (Fig. 2).

Fractionation experiments were carried out to identify the subcellular localiza-

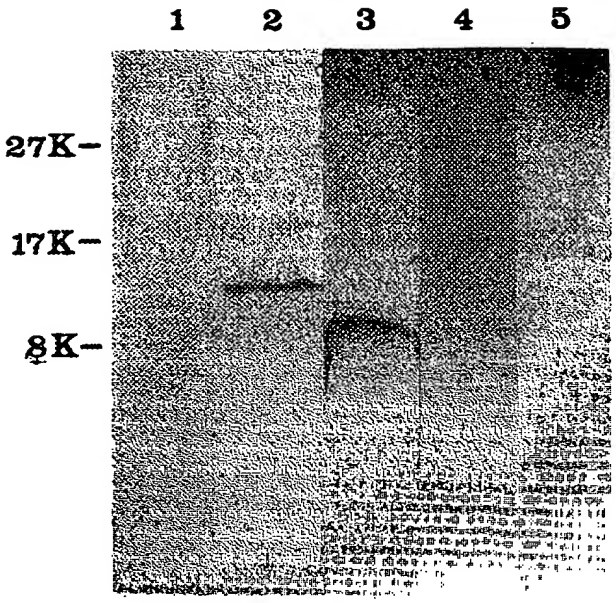


*Fig. 5.* Identification of the *vpr* gene product in MT-2 cells infected with HIV-1. MT-2 cells were infected with viruses derived from pNLEN (lane 1), pNL432 (lanes 2 and 4), or pNLSN (lanes 3 and 5), and infected-cell lysates were prepared on the appropriate day. Lysates were analyzed by Western blotting with either anti-*vpr* sera (lanes 1–3) or anti-*vpr* sera preadsorbed with the *vpr* peptide (lanes 4 and 5).

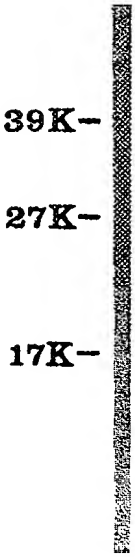


*Fig. 6.* Localization of the
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tion of *vpr* product in infected cells. The mutant virus derived from pNLEN served as a negative control. As shown in Fig. 6, the *vpr* 12-kD protein was mainly located in the membrane fraction (lane 5). No reactivity, as expected, was observed in the lysates prepared from the cells infected with the mutant virus (lanes 2, 4, and 6).

## Discussion

A major finding of our experiments presented here is the demonstration of the membrane-associated nature of the HIV-1 *vpr* 12-kD protein. Western blot analysis of subcellular fractions prepared from a cell line that constitutively produces the *vpr* gene product (Fig. 4) and from infected cells (Fig. 6) clearly shows that the *vpr* protein is located at the membrane. The slight difference in the molecular mass of the *vpr* product reported (9) probably represents a difference in the virus strain and/or of experimental conditions. This is the first report describing the subcellular localization of the HIV-1 *vpr* protein.

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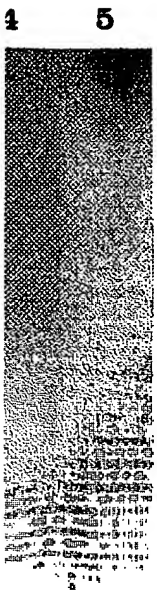


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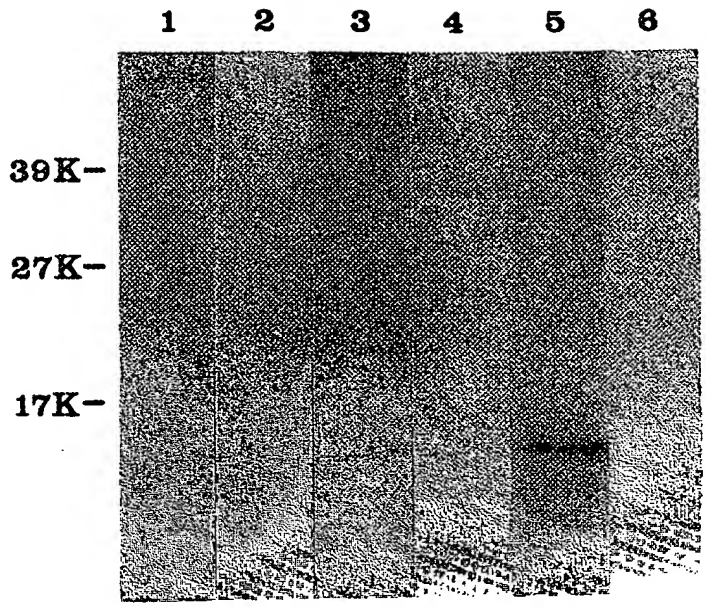


*Fig. 6.* Localization of the *vpr* gene product in MT-2 cells infected with HIV-1. MT-2 cells infected with viruses from pNL432 (lanes 1, 3, and 5) or from pNLEN (lanes 2, 4, and 6) were fractionated into subcellular components and lysates were prepared. Lysates from the nuclear fraction (lanes 1 and 2), cytosol fraction (S100; lanes 3 and 4), or membrane fraction (P100; lanes 5 and 6) were analyzed by Western blotting with the anti-*vpr* peptide sera.

Two other observations should be made here. Mutations at the *Eco*RI site (Fig. 1A) greatly reduce the production of the truncated *vpr* protein in both bacterial (Fig. 2) and mammalian (Fig. 5) cell systems. In contrast, no such effects were observed with other mutants (Figs. 2 and 5). Comparison of the predicted amino acid sequences of these mutated *vpr* proteins revealed that the meaningful difference in the amino acid residues resided in a relatively hydrophobic stretch consisting of 13 amino acids (amino acids 64–76, see Fig. 1A). Presumably, *vpr* protein, which lacks this domain, was rapidly degraded in the cells. This remains to be proved. We also noticed that the size of the *vpr* gene products expressed in bacterial and mammalian cells was exactly the same when run on the same gel (data not shown). These data suggest that the *vpr* product was not modified in mammalian cells via glycosylation and cleavage with proteolytic enzymes.

At present, the molecular mechanism by which HIV-1 *vpr* exerts its function is not completely understood. The stimulatory effect of *vpr* (8,9) is reported to act on the HIV long terminal repeat (9). Taking into account our observation that the *vpr* protein is membrane associated, the enhancing effect may not be direct. Further studies are required to elucidate the function of the *vpr* gene in detail.

**Acknowledgments**

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# Detection of I
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NOBUO YAEGASHI,[1] HA
KIICHIRO NODA,[2] AND
[1]*Department of Obstetrics*
[2]*Department of Obstetrics*

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Tohoku University of Med

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*Abstract*

DNA samples from c
cinoma of the uterine
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were found to hybrid
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52b (HPV52b), and
for HPV DNA with
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tive conditions. Aft
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and HPV18, is asso
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# Antibody Fragments Selected by Phage Display against the Nuclear Localization Signal of the HIV-1 Vpr Protein Inhibit Nuclear Import in Permeabilized and Intact Cultured Cells

A. Krichevsky,* A. Graessmann,† A. Nissim,‡[1] S. C. Piller,§ N. Zakai,* and A. Loyter*[,2]

*Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel; †Institut fur Molekularbiologie und Biochemie, Freie Universitaet Berlin, Arnimallee 22, D-14195, Berlin, Germany; ‡The Wolfson Center for Applied Structural Biology, The Hebrew University of Jerusalem, Jerusalem, Israel; and the §Center for Immunology, St. Vincent Hospital, Sydney, Australia

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**The HIV-1 Vpr protein harbors a nuclear localization signal in its N-terminal domain. A peptide bearing this domain and which is designated VprN has been used as a target to screen a phage display single chain Fv (scFv) library. Here we report the isolation of anti-VprN scFv fragments from this library. The purified scFv fragments were able to bind the VprN peptide in an ELISA-based system and to inhibit VprN-mediated nuclear import in permeabilized as well as in intact microinjected cells. Furthermore, the anti-VprN scFv fragments recognized the full-length recombinant Vpr protein and inhibited its nuclear import. The same scFv fragments did not inhibit nuclear import mediated by the nuclear localization signal of the SV40 large T-antigen demonstrating a specific effect. The use of the described inhibitory anti-VprN scFv fragments to study nuclear import of viral karyophilic proteins and their therapeutic potential is discussed.** © 2002 Elsevier Science (USA)

*Key Words:* HIV-1; Vpr; nuclear import; phage display.

## INTRODUCTION

During the last few years it has become clear that the human immunodeficiency virus type-1 (HIV-1) is able to infect terminally differentiated nondividing cells such as macrophages and quiescent T-lymphocytes. This implies that the HIV-1 genome is able to cross the intact nuclear envelope via the nuclear pore complexes (NPC) of host cells (Simm *et al.,* 1993; Lewis *et al.,* 1992). Following cell penetration, the HIV-1 particles are uncoated and the viral genome is converted to the preintegration complex (PIC). The PIC is then imported into the nuclei of infected cells by one or more of the viral karyophilic proteins (Bukrinsky *et al.,* 1992).

The ability of HIV-1 to infect nondividing cells distinguishes it from other retroviruses, such as the murine leukemia virus (MLV), that infect only proliferating cells. Indeed, PICs derived from many oncoretroviruses including MLV are nonkaryophilic and therefore enter the nucleus only during mitosis following the breakdown of the nuclear envelope (Lewis and Emerman, 1994).

In the case of HIV-1, at least three viral proteins have been proposed to be involved in nuclear import of the PIC, including the matrix (MA) protein, the viral protein of regulation (Vpr), and the viral integrase (IN) protein (Bouyac-Bertoia *et al.,* 2001; Jenkins *et al.,* 1998; Depienne *et al.,* 2000; Bukrinsky *et al.,* 1993). Thus, the nuclear import mechanisms may be partially redundant. However, the respective contribution of each of these karyophilic proteins to the nuclear import process of the viral PIC is as yet not fully understood.

The current view of the involvement of the HIV-1 karyophilic proteins in nuclear import of the PIC is based mainly on the inability of virus particles that are deleted or mutated in one of these proteins to replicate in nondividing cells (Haffar *et al.,* 2000; Koostra and Schuitemaker, 1999). However, due to the inherent limitations of these studies, the involvement of the MA and Vpr proteins for promoting nuclear import of the PIC is still controversial. Also, conflicting data have been reported on the function of the HIV-1 IN protein in mediating nuclear import of the viral PIC (Bouyac-Bertoia *et al.,* 2001; Haffar *et al.,* 2000; Petit *et al.,* 2000). Thus, the accurate role of the nuclear localization signals (NLS) elucidated within the HIV-1 MA, Vpr, and IN proteins and the function of each of these sequences in virus infection continue to be a matter of debate.

It appears that the role of Vpr in mediating nuclear import of the HIV-1 PIC is indirect and requires the coparticipation of the viral MA protein (Popov *et al.,* 1998; Haffar *et al.,* 2000). Using Vpr-deficient HIV-1 mutants, it

[1] Present address: Bone and Joint Research Unit, Barth and The London School of Medicine and Dentistry, Queen Mary, London, UK; and the Felsenstein Medical Research Center, Sackler Faculty of Medicine, Tel Aviv University, Rabin Medical Center, Belinson Campus, Petach Tikva, Israel.

[2] To whom correspondence and reprint requests should be addressed at the Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Edmond Safra Campus, Givat Ram, Jerusalem 91904, Israel. Fax: +972-2-6586448. E-mail: loyter@mail.ls.huji.ac.il.



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has been shown that in certain cell lines, such as growth-arrested T-cells, Vpr is required neither for nuclear import of the PIC nor for virus replication (Heinzinger *et al.,* 1994; Gallay *et al.,* 1997; Bukrinsky *et al.,* 1993). In such cells its role in promoting nuclear import of the viral PIC was suggested to be substituted by cellular proteins such as the Hsp70 (Agostini *et al.,* 2000). Recently, it has been reported that the Vpr protein induces transient and localized herniations in the nuclear envelope, and this process—as was suggested—provides a portal for PIC entry into the nucleus (de Noronha *et al.,* 2001).

Vpr is a small protein that is composed of 96 amino acids with a molecular weight of approximately 11 kDa (Yuan *et al.,* 1990; Baldrich-Rubio *et al.,* 2001). Several studies have shown that nuclear accumulation of this protein may be promoted by both the N- and the C-terminal domains (Jenkins *et al.,* 1998). In our previous study we used peptides derived from the Vpr to better characterize its NLS region (Karni *et al.,* 1998). We synthesized peptides corresponding to the N- (residues 17 to 34) and C-terminal (residues 77 to 96) regions and designated them as VprN and VprC, respectively. Indeed, peptides derived from the N-terminal region of Vpr, but not from its C terminus, promoted the entry of covalently attached, labeled BSA molecules into nuclei of permeabilized HeLa cells (Karni *et al.,* 1998). Our results—which were consistent with previously published observations (Jenkins *et al.,* 1998)—indicated that the Vpr protein harbors a nonconventional, negatively charged NLS in its N terminus (Karni *et al.,* 1998; Jenkins *et al.,* 1998). Nuclear import of the BSA–VprN conjugates was found to be energy and temperature dependent and was inhibited by wheat germ agglutinin (WGA), demonstrating that the observed import is an active process and occurs via the NPC.

Antibodies and peptides that specifically mask the NLSs of the HIV-1 karyophilic proteins and thus inhibit their function may offer an alternative and novel approach to elucidating the exact role of the Vpr protein as well as its relative contribution to the nuclear import process of the HIV-1 PIC. Furthermore, such studies may help in gaining a better understanding of the spatial rearrangement of the karyophilic proteins within the PIC complexes and their relative susceptibility to external ligands. This approach may also be useful for obtaining anti-viral drugs.

Here we have applied the phage display technology and used a single chain Fv (scFv) phage display library for isolation of scFv fragments against the VprN sequence. The library used in the present work is of a diversity $>10^8$ and was built by the use of a repertoire of *in vitro* rearranged human VH gene segments with random nucleotide sequences encoding the CDR3 domain (length of 4–12 residues) (Nissim *et al.,* 1994). Using this library we have been able to select more than 40 phages bearing specific anti-VprN scFvs. The present results show that the anti-VprN scFvs recognized the VprN peptide. They did not, however, recognize peptides bearing a mutated VprN or other NLSs, such as that of the SV40 large T-antigen (SV40 NLS). The anti-VprN scFv fragments strongly inhibited import mediated by the VprN peptide but not by the SV40 NLS. Furthermore, the anti-VprN scFvs were able to specifically bind to and inhibit nuclear import of a recombinant full-length Vpr–GST fusion protein.

## RESULTS

### Selection of phage particles that possess specific binding to the VprN peptide

The BSA–VprN nuclear conjugates (see Materials and Methods; Karni *et al.,* 1998) was used by us as a target for selecting anti-VprN phage particles from a semisynthetic phage display scFv library (Harrison *et al.,* 1996; Nissim *et al.,* 1994). The BSA–VprN conjugate was employed because attempts to use the free VprN peptide as a target for the library were unsuccessful (data not shown). To assure the selection of VprN-binders and to exclude BSA-binders, phages selected by the first-round panning were incubated with immunoblots coated with BSA molecules, and then unbound particles were collected (see Materials and Methods). This strategy significantly increased the percentage of specific VprN-binders, which were around 7% of the total particles screened, compared with the only about 0.5% obtained when the BSA panning step was omitted. This double selection assured the isolation of phage particles which possessed selective and specific affinity for the VprN sequence as demonstrated by ELISA tests. Only those VprN-binders which exhibited high and selective binding for BSA–VprN and not to the other NLS–BSA conjugates were considered specific binders for further analysis. BSA conjugates carrying peptides with unrelated sequences were used to detect unspecific binders (see Materials and Methods and Fig. 1).

The results in Fig. 1A show that several colonies (1–4, 11) indeed possessed specific binding to VprN, while other colonies also were able to bind other NLS–BSA conjugates in addition to VprN (colonies 6–9) or did not bind to any of the target molecules used (colonies 5, 10, 12). In our experiments over 40 specific binders, namely phages which bind to VprN exclusively and not to other NLSs, were selected (not shown). Quantitative estimation of the binding abilities of three phage colonies (designated as phages A, B, C), which were chosen for their strong and specific binding to the target sequence, are

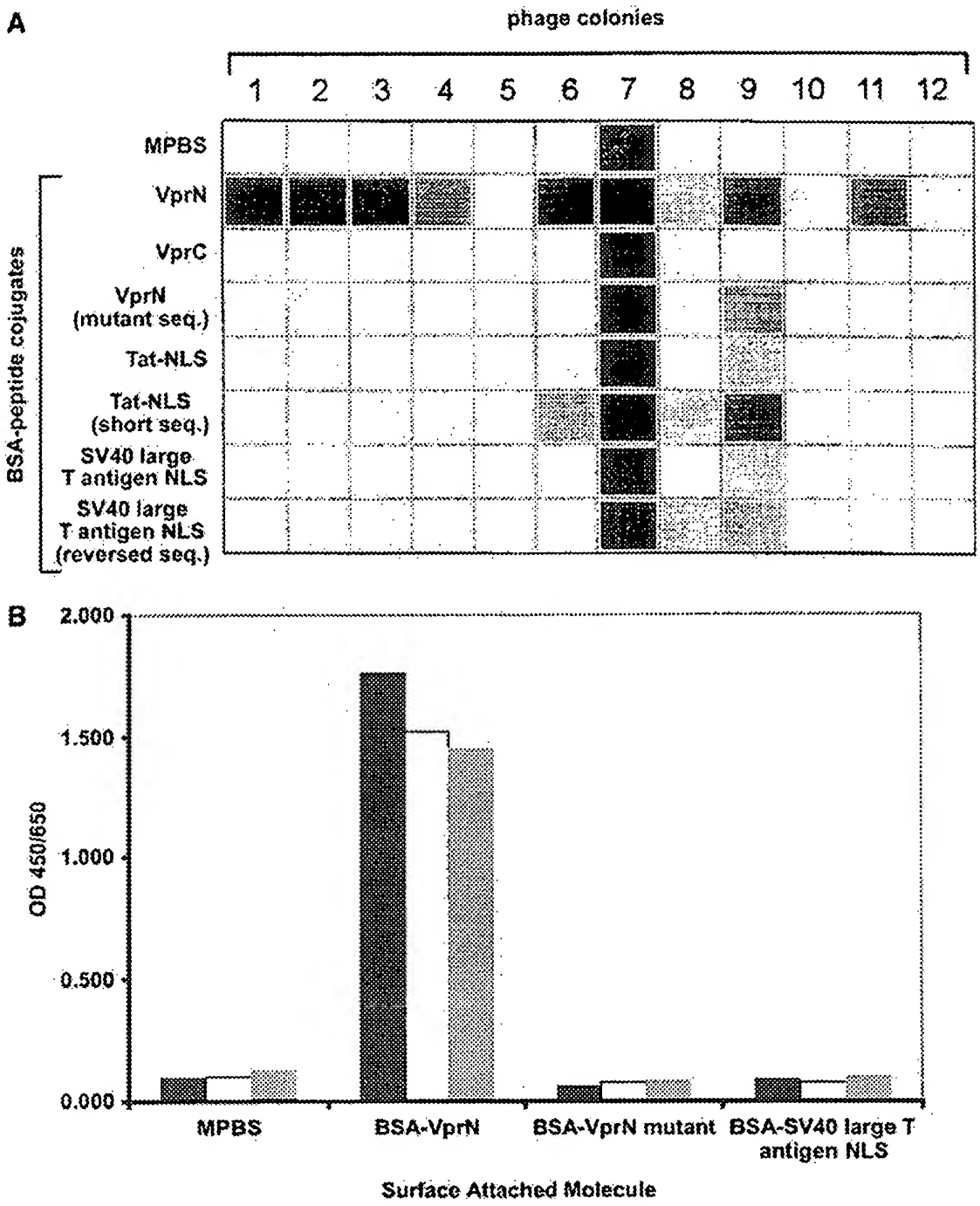


FIG. 1. Binding of selected phage colonies to the BSA–VprN conjugate. (A) Binding was estimated by the ELISA assay system (see Materials and Methods). Numbers indicate individual phage colonies. The OD values were determined by an ELISA reader and converted to a gray scale. Note that black and white fields indicate strong and no binding, respectively; gray fields indicate binding of intermediate strength. Also note that phages 1–4 as well as 11 exhibited strong and specific binding to the VprN sequence. (B) Specific binding of the three selected phage colonies used in the present work to the BSA–VprN conjugate (▩, phage A; □, phage B; ▨, phage C). As can be seen, all three phage preparations specifically bound the BSA–VprN conjugate, but did not react with the BSA–VprN mutant or the BSA–SV40 NLS. All experimental conditions were as described under Materials and Methods. MPBS, milk (skimmed milk 2%) in PBS.

shown in Fig. 1B. These three colonies showed binding abilities to the VprN about 10-fold higher than those to its mutant (which differs from the wild-type peptide only by three amino acids; see Materials and Methods) or to an unrelated NLS, such as the SV40 NLS.

### Anti-VprN scFv fragments possess high and specific binding abilities to the VprN peptide

Highly purified scFvs with the appropriate molecular weight (MW of 30 kDa) were obtained from phages A, B,

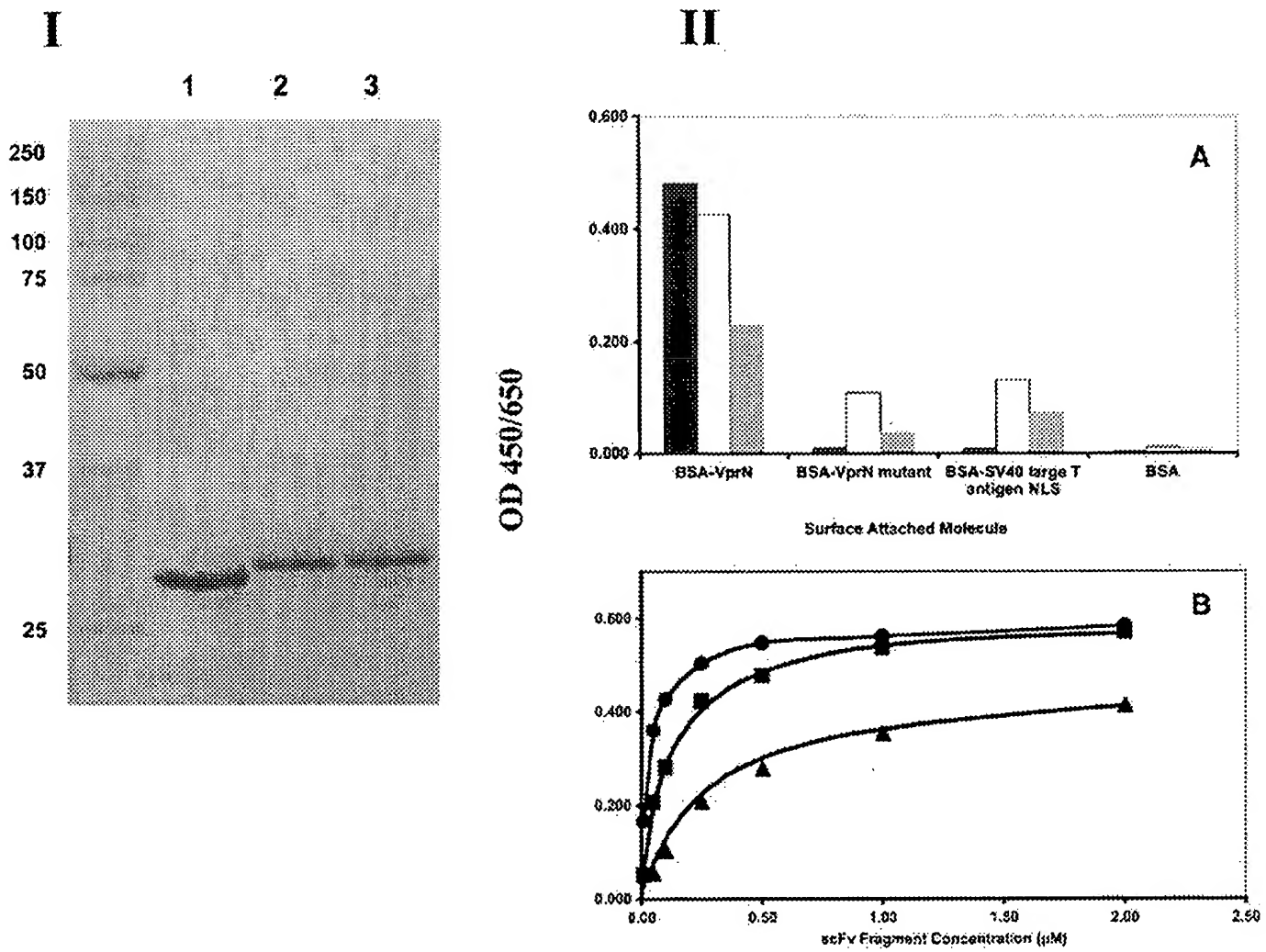


FIG. 2. [I] SDS–PAGE of the purified anti-VprN scFvs. scFv fragments 1, 2, and 3 (lanes 1–3, respectively) were analyzed using a 12% acrylamide gel. Please note that fragment 1 is of the lower molecular weight due to the fact that its random CDR3 insert contains only 4 amino acids compared to the 10 amino acids of fragments 2 and 3. [II] Interaction of the anti-VprN scFv fragments with the BSA–NLS conjugates (A) Binding of fragments 1, 2, and 3 to various BSA–NLS conjugates: ■, fragment 1; □, fragment 2; ■, fragment 3. (B) The effect of the scFv fragment concentrations on binding to BSA–VprN: ●, fragment 1; ■, fragment 2; ▲, fragment 3. Binding was estimated using the ELISA system as described under Materials and Methods.

and C following the use of Sepharose beads with chemically attached BSA–VprN conjugates and were designated fragments 1, 2, and 3, respectively (Fig. 2[I] and see Materials and Methods).

Binding studies revealed that all three purified scFv fragments were able to specifically bind to the BSA–VprN conjugate but failed to show any significant binding to the BSA (nonconjugated) molecules, indicating specific attachment to the VprN moiety of the conjugate (Fig. 2[II,A]). Binding was saturable, reaching maximum values at a concentration of 1 $\mu$M with fragments 1 and 2 and at 2 $\mu$M with fragment 3 (Fig. 2[II,B]). The highest binding specificity and binding values were exerted by fragment 1, which showed an about 100-fold higher binding to the BSA–VprN conjugate than to conjugates bearing the VprN mutant or the SV40 NLS (Fig. 2[II,A]). The binding specificities of fragments 2 and 3 were somewhat less pronounced. Both exhibited, in addition to the high binding values to VprN, some low, yet detectable binding to the VprN mutant and to the SV40 NLS (Fig. 2[II,A]).

Binding of the three scFv fragments to their antigen, the VprN(−BSA) molecule, was inhibited by the addition of soluble BSA–VprN conjugates or free VprN peptides (Fig. 3). However, the BSA–VprN conjugates competed with and blocked the specific binding of the scFvs much better than the free VprN peptides (Fig. 3). As can be seen, binding of fragment 1 to its antigen was inhibited up to 90% in the presence of about 5 $\mu$M BSA–VprN, while more than 15 $\mu$M was needed to exert the same inhibition by the free VprN peptide. The high inhibitory effect of the BSA–VprN conjugate—compared to that of the free VprN peptide—can be attributed to the fact that each BSA molecule bears about

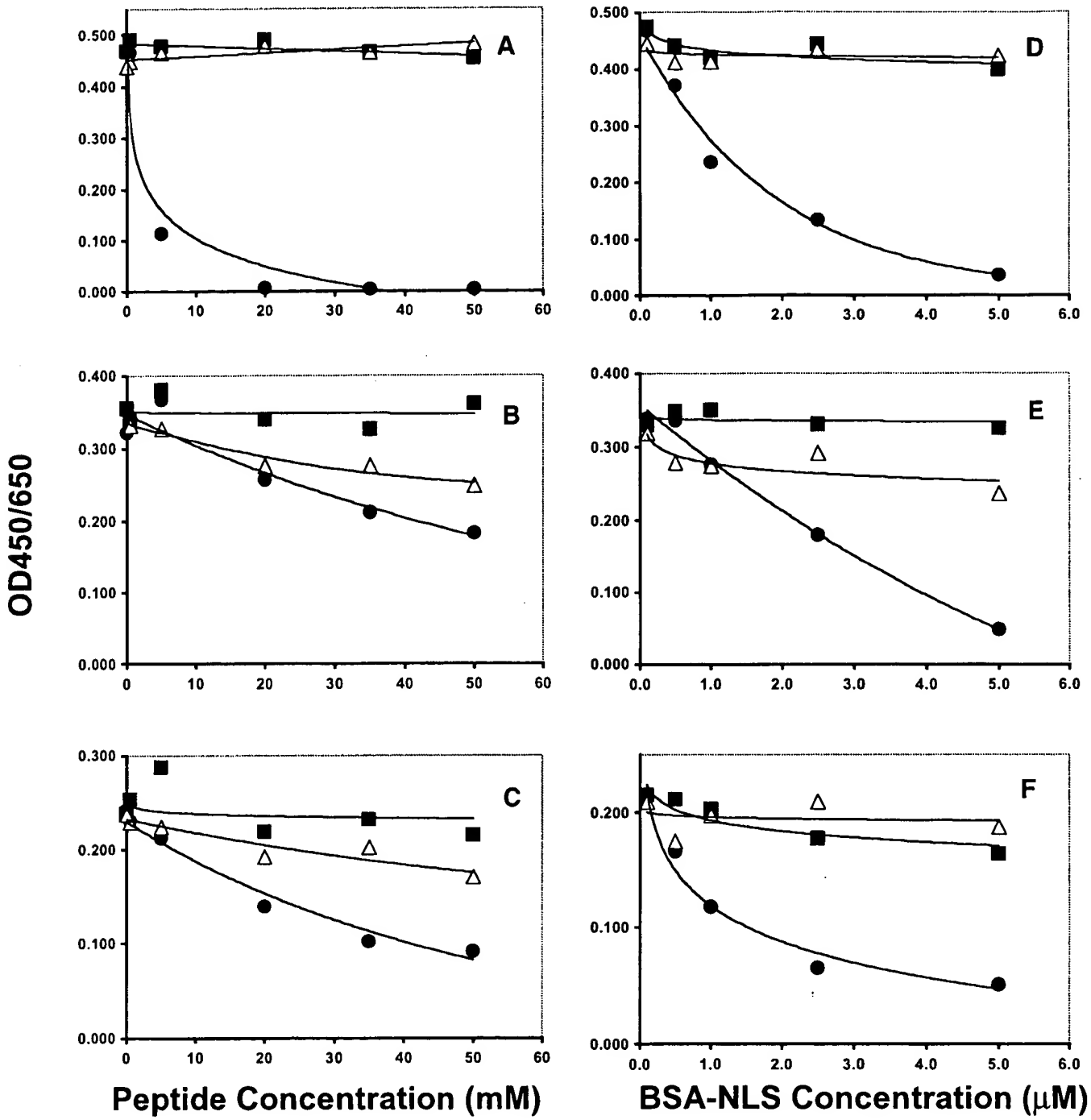


FIG. 3. Binding of the anti-VprN scFv fragments to a surface-bound BSA–VprN conjugate: specific inhibition by soluble BSA–VprN conjugate and VprN peptide. BSA–peptide conjugates and free peptides were mixed with fragments 1–3 at the indicated increasing concentrations. The mixture obtained was added to surface bound BSA–VprN conjugates. Following incubation for 2 h at 37°C, binding of the scFv fragments to the surface-attached BSA–VprN was estimated by the ELISA assay system as described under Materials and Methods. ●, BSA–VprN conjugate or the VprN peptide; △, BSA–SV40 NLS conjugate or the SV40 NLS peptide; and ■, BSA–VprN mutant conjugate or the VprN mutant peptide. A, D, fragment 1; B, E, fragment 2; C, F, fragment 3.

three or four covalently attached VprN peptides (Friedler *et al.*, 1999). No or low inhibition was observed by the addition of a peptide bearing the sequence of the VprN mutant, by its BSA conjugate, or by the free SV40 NLS peptide or its conjugate (Fig. 3).

### Inhibition of VprN-mediated nuclear import by anti-VprN scFv fragments

The results in Figs. 4[I] and 4[II] show that the addition of purified anti-VprN fragments to the *in vitro* nuclear

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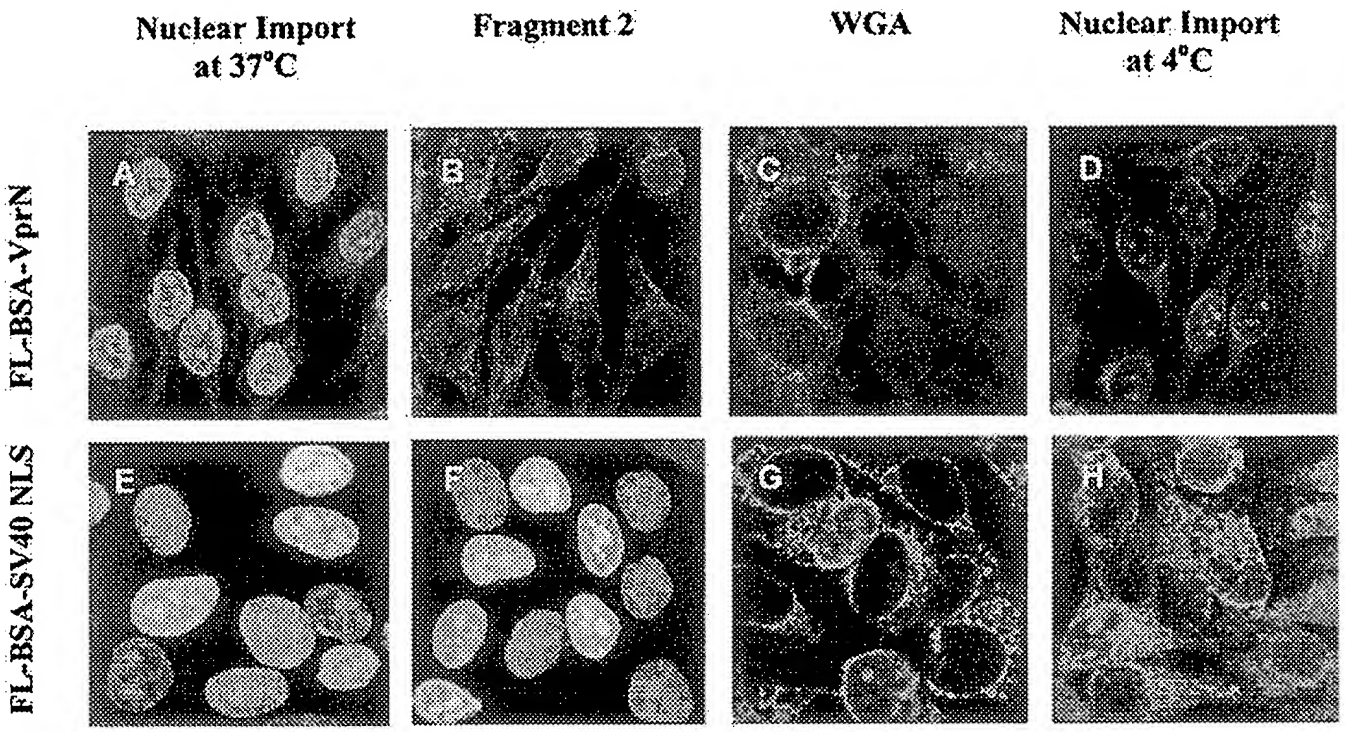
Nuclear Import at 37°C
Fragment 2
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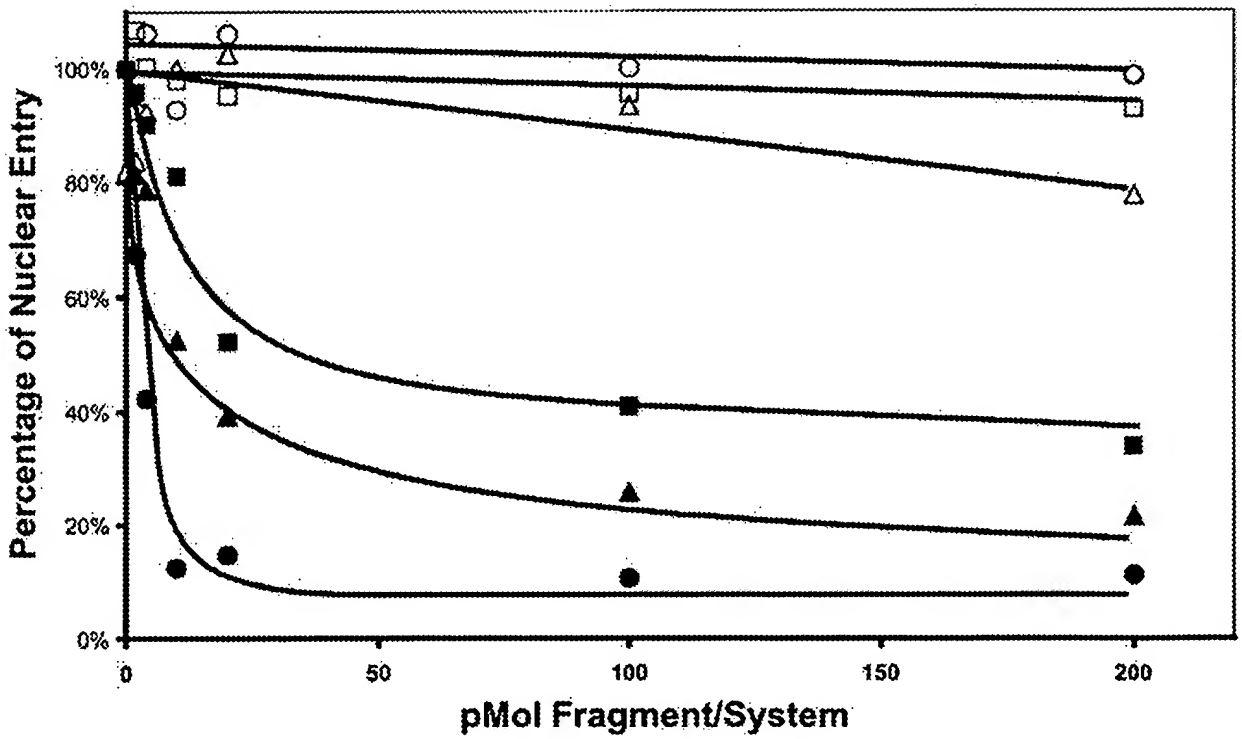
Nuclear Import at 4°C
FL-BSA-VprN
FL-BSA-SV40 NLS
A
B
C
D
E
F
G
H

II


Percentage of Nuclear Entry
100%
80%
60%
40%
20%
0%
0
50
100
150
200
pMol Fragment/System

import assay system inhibited VprN-mediated nuclear import. Inhibition of nuclear import was also observed when fragment 2 was comicroinjected with BSA–VprN into intact cultured COS7 cells (Fig. 5). Anti-VprN fragment 2 was selected for most of the experiments in which inhibition of nuclear import was studied since it showed the highest binding activity to the recombinant Vpr–GST and high binding activity to BSA–VprN (see Fig. 6A). However, the inhibitory effects obtained when fragment 1 or 3 was applied (see Fig. 4[II]) were very similar to those obtained with anti-VprN fragment 2.

The photographs in Fig. 4[I] demonstrate the inhibition of nuclear import of fluorescently labeled BSA–VprN (FL-BSA–VprN) conjugates in permeabilized HeLa cells by fragment 2. The specificity of the scFv fragments can be inferred from the results showing that no inhibition was observed when BSA conjugates bearing a different NLS, such as that of the SV40 NLS, were used as the transport substrate (Fig. 4[I]B, F). Nuclear import of the BSA–VprN conjugate was characterized by the same features that characterize specific and active nuclear import, namely, like the nuclear import of BSA–SV40 NLS, it was inhibited by the addition WGA and did not occur at 4°C (Fig. 4[I]C, D, G and H) (Goldfarb *et al.*, 1986; Karni *et al.*, 1998). Quantitative estimation of nuclear import using an ELISA-based assay system (Karni *et al.*, 1998) revealed that the inhibition observed by the three anti-VprN fragments was between 70 and 90% and close to 0% when the BSA–VprN and BSA–SV40 NLS conjugates were used as transport substrates, respectively (Fig. 4[II]).

Similar results were obtained when the effect of the scFv fragments was studied within the environment of intact cells. As can be seen in Fig. 5, microinjected FL-BSA–VprN as well as fluorescently labeled BSA–SV40 NLS (FL-BSA–SV40 NLS) conjugates readily accumulated within the intranuclear space of the microinjected cells (Figs. 5A and 5B). No nuclear import was observed when labeled BSA molecules lacking an NLS peptide were microinjected (Fig. 5E). However, when the same transport substrates were comicroinjected with fragment 2, complete inhibition of nuclear import was observed in the cells microinjected with the FL-BSA–VprN (compare Fig. 5C to Fig. 5D).

### The anti-VprN scFv fragments block nuclear import of the full-length recombinant Vpr–GST fusion protein

The scFv fragments selected by the use of the BSA–VprN conjugate as a target also recognized a full-length recombinant Vpr–GST fusion protein. Binding of fragments 2 and 3 to the Vpr–GST fusion protein was very close to that observed to the BSA–VprN conjugate (Fig. 6A). Fragment 1 showed somewhat lower binding abilities to the recombinant protein compared to the binding observed with the BSA–VprN conjugate (Fig. 6A). Specificity of binding can be inferred from the results shown in Figs. 6B and 6C. Binding of all three scFv fragments to the Vpr–GST was strongly inhibited by the addition of the VprN peptide (Fig. 6B) as well as of the BSA–VprN conjugate (Fig. 6C). No or marginal inhibition was observed when the VprN mutant peptide or its BSA conjugate was added (Figs. 6B and 6C). These results strongly indicate that the domain with which the scFv fragments interact within the full-length Vpr protein is the VprN, namely the domain which includes amino acids 17–34 (Karni *et al.*, 1998). Figure 6 also shows that to inhibit the binding of the scFv fragments to the Vpr–GST, much lower concentrations of the BSA–VprN conjugate were required compared to those of the VprN peptide. This observation is consistent with the results obtained by the competition experiment shown in Fig. 3.

Inhibition of the nuclear import of the fluorescently labeled recombinant Vpr–GST fusion protein by fragment 2 is demonstrated in Fig. 7. The same results were obtained using fragments 1 and 3 (not shown). The view that the nuclear import of the recombinant Vpr–GST protein is receptor mediated and occurs via the nuclear pore complex can be inferred from the results showing that it was inhibited by WGA—the addition of which blocks active nuclear import (Adam *et al.*, 1990)—and by unlabeled Vpr–GST fusion protein (Figs. 7C and 7D). Nuclear import of Vpr–GST was characterized by the same features that characterize active nuclear import; namely, it was ATP dependent, it was partially inhibited by GTP-γ-S, and its extent was significantly reduced following incubation at 4°C (not shown).

FIG. 4. [I] Inhibition of VprN-mediated nuclear import in permeabilized HeLa cells by anti-VprN scFv fragment 2. Nuclear import was followed by fluorescence microscopy using FL-BSA–VprN (A) and FL-BSA-SV40–NLS (E) as transport substrates; (B) and (F) as in (A) and (E) but in the presence of fragment 2 at a molar ratio of scFv:transport substrate of 2:1; (C) and (G) as in (A) and (E) but in the presence of WGA (25 μM); (D) and (H) as in (A) and (E), but nuclear import was performed at 4°C. As can be seen, nuclear import is observed in (A, E, and F) but not in (B–D, G, and H). It should be noted that although the results in the figure demonstrate specific inhibition at a relatively low scFv:transport substrate ratio (2:1), the specificity of inhibition was achieved also at higher ratios of at least up to 20:1. [II] Inhibition of VprN-mediated nuclear import by anti-VprN scFv fragments: quantitative estimation. Nuclear import assay systems contained 1.5 μg (~20 pmol) of biotinylated BSA–VprN and 0.75 (~10 pmol) μg of biotinylated BSA–SV40 NLS conjugates. A volume of 3–4 μl containing increasing concentrations of fragment 1, 2, and 3 in PBS was added to the transport substrates, and the mixture obtained was applied to digitonin-permeabilized Colo-205 cells. Nuclear import of the biotinylated BSA–NLS conjugates was estimated by the ELISA-based method (Karni *et al.*, 1998; Melchior *et al.*, 1993, and Materials and Methods). Each nuclear import experiment was repeated at least three times. The data given represent results obtained from one single experiment. Quantitative differences between repeated experiments never exceeded ±20%. White symbols designate biotinylated BSA–SV40 NLS conjugates; black symbols designate biotinylated BSA–VprN conjugates; ● or ○, anti-VprN fragment 1; ▲ or △, anti-VprN fragment 2; ■ or □, anti-VprN fragment 3.

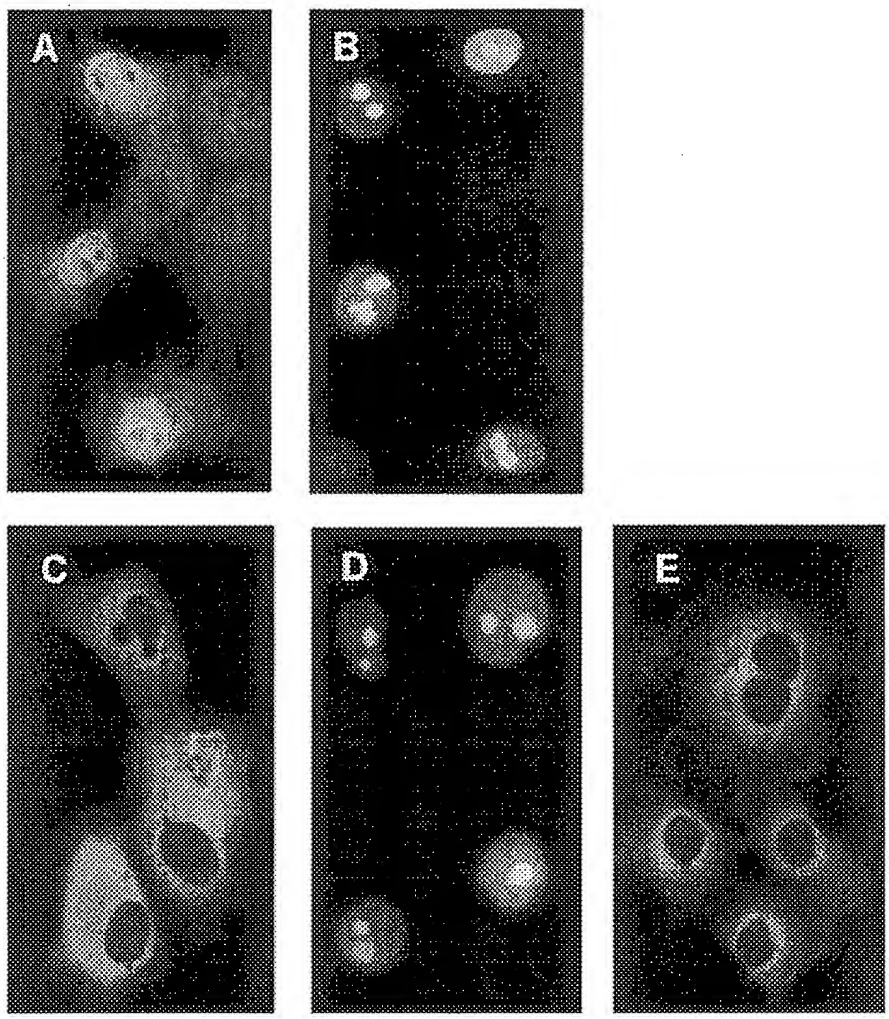
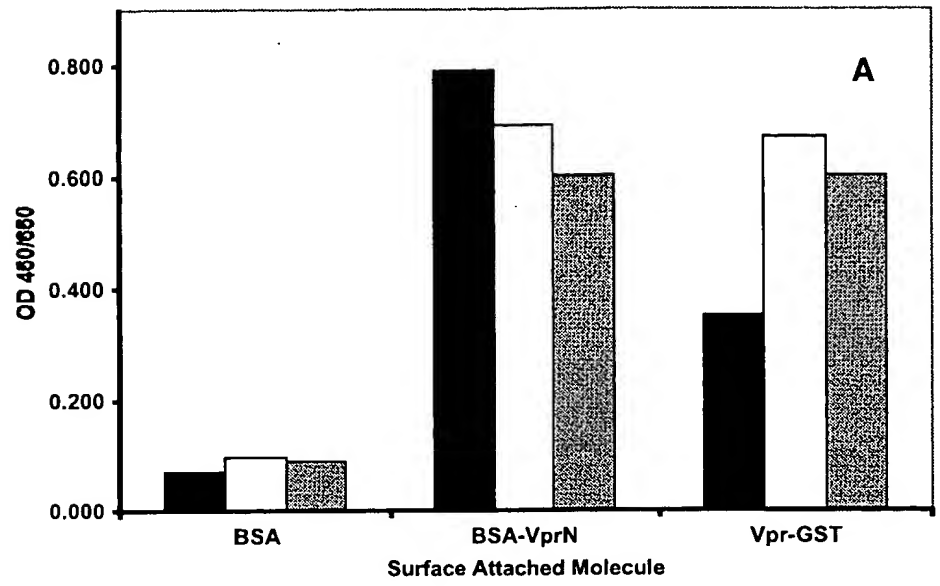


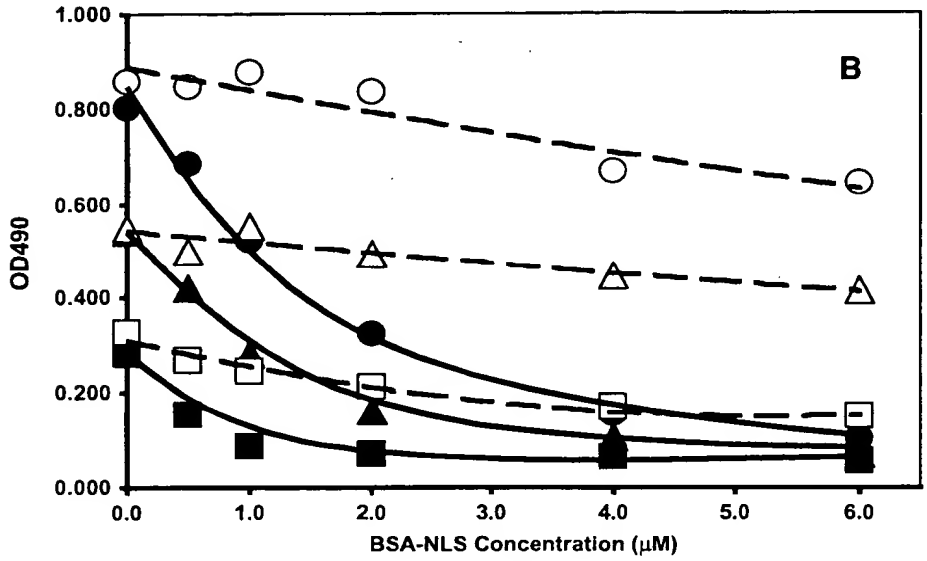

FIG. 5. Inhibition of VprN-mediated nuclear import in microinjected COS7 cells by anti-VprN scFv fragment 2. Cultured COS7 cells were microinjected (Graessmann and Graessmann, 1983) with $2\text{–}3^{(-10)(-11)}$ ml of solutions containing the following: (A) FL-BSA–VprN, (B) FL-BSA–SV40 NLS, (C) a mixture of FL-BSA–VprN with fragment 2 at a 1:2 molar ratio, (D) FL-BSA–SV40 NLS with fragment 2 at the same ratio as in (C), (E) FL-BSA. Please note nuclear import in (A, B, and D) but not in (C and E).

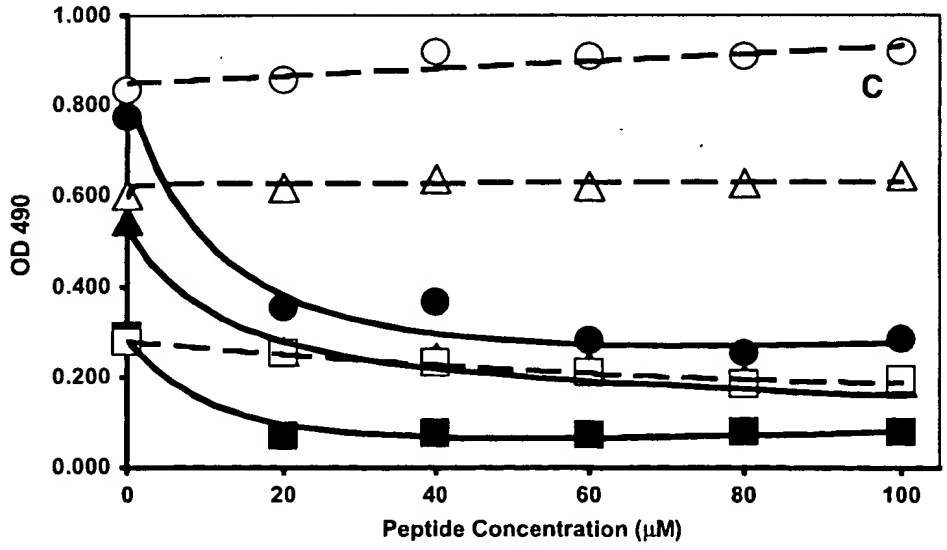
### A synthetic peptide derived from the CDR3 insert of an anti-VprN scFv fragment binds the BSA–VprN conjugate but fails to inhibit its nuclear import

It is possible that the binding to the VprN moiety and the inhibition of nuclear import exerted by the scFv fragments may be promoted, fully or partially, by the random amino acids inserted into the CDR3 loop of the scFv domain (Nissim *et al.,* 1994). It was of interest, therefore, to elucidate the amino acid sequences of this insert and to study the binding abilities of synthetic peptides carrying these sequences. The corresponding amino acid sequences encoded by the variable insert in the VH-CDR3 region of the scFv in the 40 anti-VprN phages isolated were determined (not shown). However, no consensus sequence could be elucidated between the various variable insert sequences determined using the Clastal W (Thompson *et al.,* 1994) and MEME (Bailey and Elkan, 1994) algorithms.

FIG. 6. Binding of the anti-VprN scFv fragments to the Vpr–GST fusion protein. (A) Binding of the scFv fragments to surface-bound BSA, to VprN–BSA conjugate, and to the Vpr–GST fusion protein was estimated using the ELISA assay system as described under Materials and Methods. ■, fragment 1; □, fragment 2; ● (gray), fragment 3. (B, C) Binding of the anti-VprN scFv fragments to the full-length Vpr–GST protein: specific inhibition the by VprN–BSA conjugate and by the VprN peptide. BSA–VprN or BSA–VprN mutant conjugates (B) and VprN or VprN mutant peptides (C) were added to the scFv fragment solutions at the indicated concentrations. The mixture obtained was added to surface-bound the Vpr–GST protein. Following 2 h of incubation at 37°C, binding of the scFv fragments to Vpr–GST was estimated by ELISA as described under Materials and Methods. Black symbols and solid lines: BSA–VprN conjugate or VprN peptide; white symbols and dashed lines: BSA–VprN mutant conjugate or VprN mutant peptide. ■ or □, fragment 1; ● or ○, fragment 2; ▲ or △, fragment 3.


A
0.800
0.600
0.400
0.200
0.000
OD 450/650
BSA
BSA-VprN
Vpr-GST
Surface Attached Molecule


B
1.000
0.800
0.600
0.400
0.200
0.000
OD490
0.0
1.0
2.0
3.0
4.0
5.0
6.0
BSA-NLS Concentration (μM)


C
1.000
0.800
0.600
0.400
0.200
0.000
OD 490
0
20
40
60
80
100
Peptide Concentration (μM)

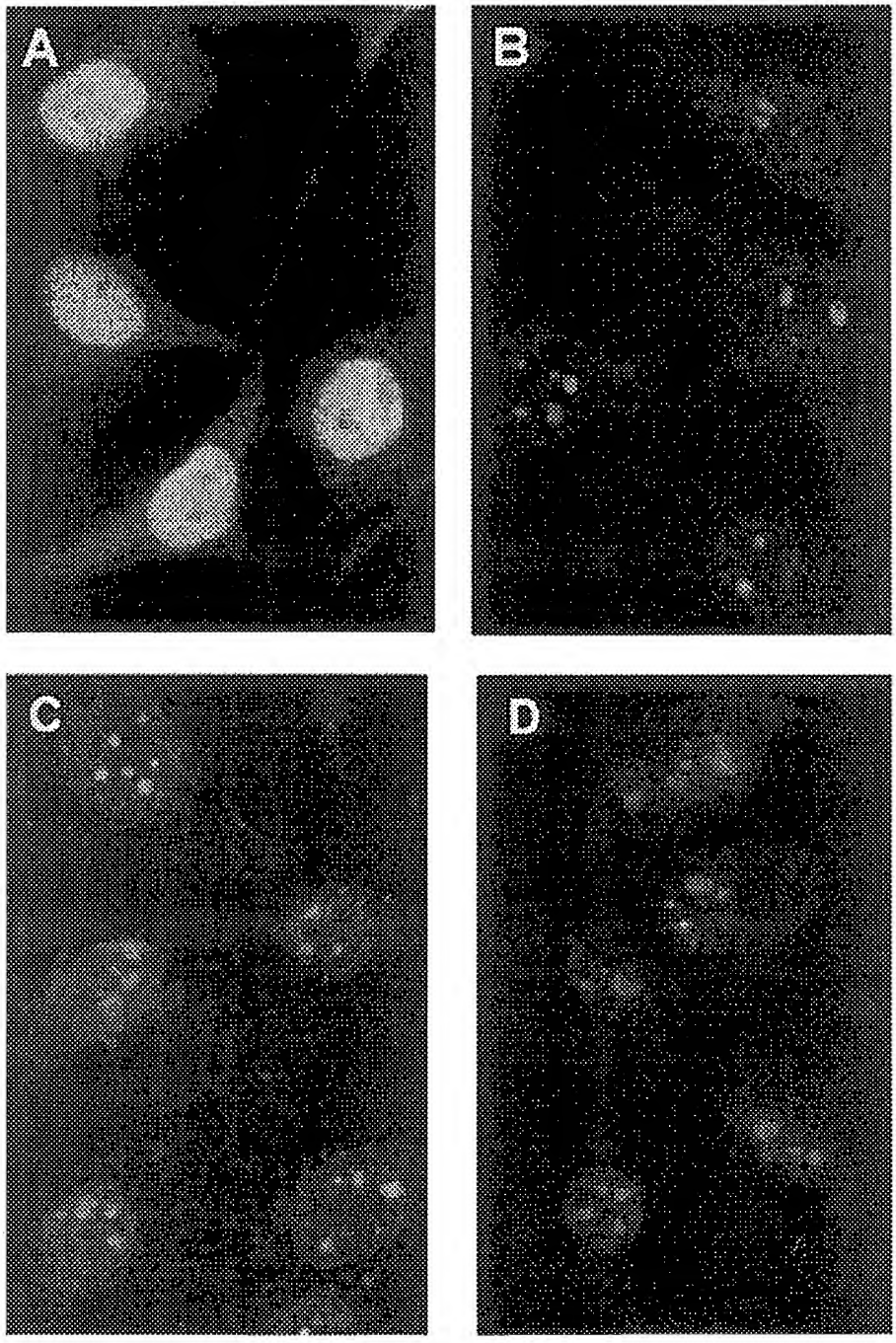



FIG. 7. Inhibition of nuclear import of FL-Vpr–GST by scFv anti-VprN fragment 2. Recombinant Vpr–GST was obtained, fluorescently labeled, and imported into nuclei of digitonin of permeabilized HeLa cells as described under Materials and Methods. (A) FL-Vpr–GST; (B) FL-Vpr–GST mixed with fragment 2 in a mole/mole ratio of 1:4; (C) FL-Vpr–GST mixed with WGA (25 $\mu$M); and (D) FL-Vpr–GST in the presence of fivefold unlabeled Vpr–GST.

The amino acid sequences of the inserts within scFv fragments 1–3 are shown in Fig. 8A. Peptides corresponding to these inserts have been synthesized and designated as peptides a, b, and c, respectively (see Fig. 8A). Of the three synthetic peptides, only peptide b exhibited specific binding to the BSA–VprN conjugate, as was determined by the ELISA assay system (Figs. 8B and 8C). Peptides a and c, the sequences of which are based on the inserts within the two other BSA–VprN binders (A and C), failed to bind this conjugate (Figs. 8B and 8C). Binding of peptide b to the VprN sequence was saturable and reached maximum values at a peptide concentration of 1 mM (Fig. 8C). However, its binding to VprN was only about two- to threefold higher than that observed with the VprN mutant or the SV40 NLS, implying that the interaction between peptide b and VprN is of low affinity (Fig. 8B).

A

| Sequence | Corresponding phage | Peptide designation |
|---|---|---|
| ISSD | A | a |
| AFMKSGKRFIH | B | b |
| HFHYKGKLQTF | C | c |

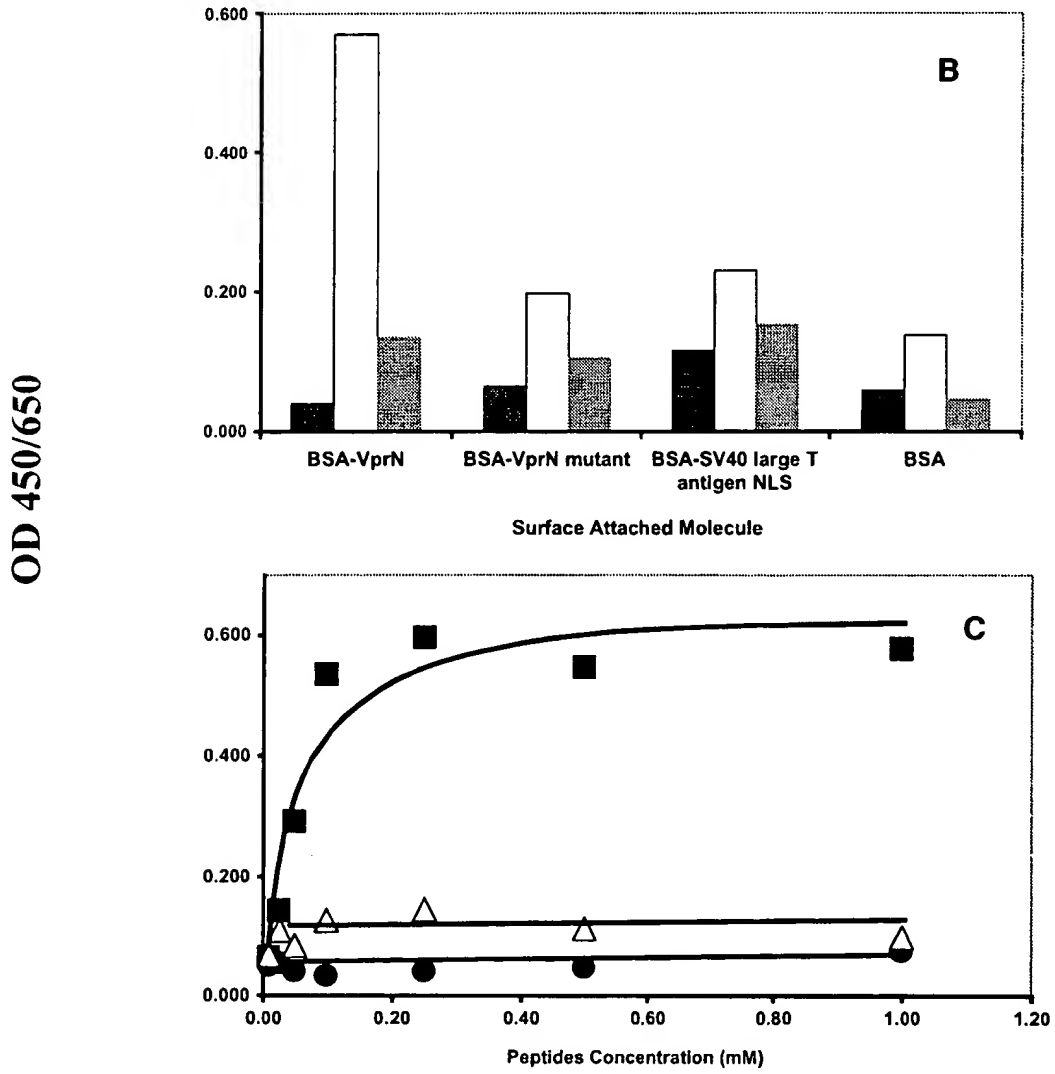



FIG. 8. Interaction of the anti-VprN peptides with the BSA–NLS conjugates. (A) The oligonucleotide sequences of the three random inserts in the VH–CDR3 loop. (B) Estimation of the binding abilities of the anti-VprN peptides to various BSA–NLS conjugates (▨, peptide a; □, peptide b; ▨, peptide c). (C) Dependency of the binding to the BSA–VprN conjugate on the anti-VprN peptide concentrations (●, peptide a; ■, peptide b; △, peptide c).

However, even at relatively high concentrations, the addition of peptide b did not cause any significant reduction in the nuclear import of fluorescently labeled BSA–VprN conjugates into the nuclei of permeabilized HeLa cells (not shown).

## DISCUSSION

The role that the Vpr protein plays in promoting nuclear import of the HIV-1 PIC is unclear and controversial (Heinzinger *et al.,* 1994; Popov *et al.,* 1998; Gallay *et al.,* 1997; Agostini *et al.,* 2000). The use of the presently described inhibitory anti-VprN scFvs may offer a new approach to the study of the requirement of the Vpr protein for allowing HIV-1 infection in nondividing cells and for the elucidation of the role of the Vpr protein in nuclear import of the viral PIC. In addition, inhibition of nuclear import of viral karyophilic proteins by specific anti-NLS molecules may lead to the development of a novel way to combat virus infection in general and the AIDS disease in particular.

In the present work we have used a phage display scFv library to obtain specific anti-NLS scFv fragments. BSA molecules with covalently attached VprN peptide, a peptide bearing the NLS of the HIV-1 Vpr protein, have been used as a target for the phage display libraries. The relatively high percentage of VprN binding phages selected by the use of this conjugate as a target may be due to the fact that each molecule of the BSA carrier bears, on average, three to four covalently attached VprN molecules (Friedler *et al.,* 1999). Furthermore, the phage particles of the library used possess at the most one antibody fragment per particle, thus allowing the selection of antibodies with relatively high affinities (Nissim *et al.,* 1994; Harrison *et al.,* 1996).

The choice of the VprN peptide as a target was based on the assumption that it harbors the HIV-1 Vpr NLS, since this peptide was shown to promote nuclear import of conjugated BSA molecules as well as of $\beta$-galactosidase (Karni *et al.,* 1998; Jenkins *et al.,* 1998). This synthetic peptide, which consists of the amino acids 17–34 from the N-terminal domain of the HIV-1 Vpr protein, has been suggested to possess an $\alpha$-helical structure, probably similar to that within the intact protein (Luo *et al.,* 1998; Wecker and Roques, 1999). Bearing a relatively stable $\alpha$-helical secondary structure makes the VprN peptide a convenient target for phage display libraries and for obtaining complementary scFvs or peptides. Indeed, the three anti-VprN scFv fragments analyzed in this study possessed high and specific binding activity to the VprN peptide. The scFvs obtained also interacted with the full-length recombinant Vpr–GST protein, most likely recognizing its VprN domain. This can be inferred from the results showing that the interaction between the scFv fragments and the recombinant Vpr–GST protein could be inhibited by the VprN peptide and by its BSA conjugate.

It is our view that the most interesting and significant observation of the present work is the ability of the anti-VprN scFvs to inhibit nuclear import mediated by the VprN peptide or of the recombinant Vpr–GST fusion protein. Quantitative estimation, using an ELISA-based system, revealed that the inhibition degree was highly dependent on the mole/mole ratios between the scFv fragments and the VprN. At high molar ratios almost total inhibition of nuclear import was observed.

The ability of the scFvs to bind to the VprN sequence as well as to inhibit nuclear import was highly specific. VprN-mediated but not SV40 NLS-mediated nuclear import was inhibited. Altogether these results strongly indicate that the inhibition observed is due to specific masking of the VprN moiety by the scFv fragments and thus prevention of interaction with its putative cellular receptors. In addition, the inhibition observed within the microinjected cells suggests that the intracellular environment did not promote dissociation of the scFv fragment–VprN complex, indicating a high affinity between these two proteins.

The inhibition of nuclear import obtained in the present work by the three anti-VprN scFvs strongly indicates that the VprN domain indeed mediates nuclear import of the intact Vpr protein. The current view that the VprN domain is required for the nuclear import is based mainly on experiments showing that peptides bearing its sequence promoted nuclear import of nonkaryophilic proteins (Jenkins *et al.,* 1998; Karni *et al.,* 1998). Our present results, demonstrating the ability of the anti-VprN scFvs to totally block nuclear import of the full-length protein, support the view that the VprN domain is absolutely required for nuclear import of the Vpr protein. It has also been suggested that the C-terminal domain of the Vpr bears an NLS sequence that contributes to its karyophilic properties (Jenkins *et al.,* 1998). However, in our previous experiments a synthetic peptide bearing an amino acid sequence from the C terminus of the Vpr protein (residues 77–96) failed to mediate nuclear import of covalently attached BSA molecules (Karni *et al.,* 1998). Thus, the present results further support our previous observations indicating that only the N terminus contributes to the karyophilic properties of this protein. However, in the present work the Vpr protein was fluorescently labeled by covalent attachment of a rhodamine–maleimide molecule to the cysteine residue located at the C terminus of this protein (amino acid 76) (Baldrich-Rubio *et al.,* 2001), as well as to the additional cysteine residues located in the GST moiety of the Vpr–GST protein. Therefore, the possibility that the chemical modification of the cysteine residue located at the C terminus (amino acids 73–96) (Jenkins *et al.,* 1998) caused inacti-

vation of the putative NLS at this domain cannot be excluded. Moreover, the binding of the relatively high-molecular-weight anti-VprN scFv (30 kDa) fragment to the relatively small Vpr protein (11 kDa) may cause masking of most of the Vpr molecule, including the putative C-terminal NLS. Also, the GST fusion at the C terminus itself may mask the putative C-terminal NLS. Thus, our present results do not completely exclude the possibility that an additional domain within the Vpr protein, besides the VprN, may contribute to its karyophilic properties.

Here we have used an isolated peptide, the VprN peptide, and not the full-length protein as a target for selecting anti-VprN scFv fragments. This certainly is preferable, especially in cases in which the availability of highly purified proteins is limited but the domains of interest and their amino acid sequences within such proteins are known. Furthermore, by the use of an intact protein as a target for a phage display library, the generation of antibodies or peptides complementary to various epitopes and not necessarily to the desired domains cannot be avoided. Our present results clearly demonstrate that the scFv fragments selected by the use of isolated peptide as a target recognized the same sequence within the intact protein. The drawback of this approach is that isolated peptides cannot be employed as a target when the desired epitope is discontinuous within the intact protein. However, when the detailed 3D structure of such discontinuous epitopes is known, backbone cyclic peptides bearing the corresponding structure can be designed and synthesized. Such cyclic peptides can then be used as a target for the phage display library, avoiding again the use of the full-length protein (Kasher *et al.,* 1999).

Cell-permeable anti-NLS inhibitory peptides may potentially offer an alternative system to that of the scFv fragments for study of the role of NLS domains in virus infection. Such inhibitory peptides may also serve as lead molecules for the development of anti-viral and anti-HIV drugs. Therefore, attempts were made in the present work to obtain synthetic peptides the amino acid compositions of which were based on the sequences of the CDR3 inserts of the three selected inhibitory scFvs. However, the peptides obtained either failed to bind the target VprN peptide or showed low binding affinities compared to that of the anti-VprN scFvs. The binding abilities of the anti-VprN peptides may be increased by error-prone PCR and mutagenesis techniques (Miyazaki *et al.,* 1999; Saviranta *et al.,* 1998; Harayama, 1998; Ling and Robinson, 1997) or alternatively by the use of phage display peptide libraries. Anti-VprN peptides that will block nuclear import of the viral PIC can serve as lead compounds for the obtaining of low-molecular-weight anti-Vpr molecules which potentially can be used for inhibition of HIV-1 infection.

## MATERIALS AND METHODS

### Cell cultures

Colo-205 (human colon adenocarcinoma, ATCC CCL 222), COS7, and HeLa cells were maintained in RPMI 1640 (Colo-205 cells) and DMEM (COS7 and HeLa cells) supplemented with 10% FCS, 0.3 g/L L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin (Beit Haemek Ltd., Kibbutz Beit Haemek, Israel), as previously described (Broder *et al.,* 1997).

### Synthesis of peptides

The following peptides were synthesized using a previously described method (Karni *et al.,* 1998): SV40 large T-antigen NLS (SV40 NLS, $^{126}$PKKKRKV$^{132}$C) (Kalderon *et al.,* 1984), a peptide bearing the SV40 large T-antigen NLS in reverse order ("revertant") (C$^{132}$VKRKKKPG$^{126}$), a peptide derived from the N terminus of the Vpr protein (VprN,C$^{16}$NEWTLELLEELKNEAVRHF$^{34}$) and one from its C terminus (VprC,C$^{77}$RHSRIGVTRQRRARNGASRS$^{96}$), VprN mutant (C$^{16}$NEATLELLPELKNPAVRHF$^{34}$) (Karni *et al.,* 1998), the HIV-1 Tat-NLS (the ARM sequence, C$^{48}$GRKKRRQRRRAHQN$^{61}$), and a Tat short NLS (C$^{48}$GRKKR$^{52}$) (Friedler *et al.,* 2000). Cysteine residues were added to the N or to the C terminus of the original NLS sequences. Peptides derived from the CDR3 random insert region of anti-VprN scFv fragments (for sequences see Fig. 8C) were synthesized using the same procedure, and when needed a biotin molecule was added during the synthesis to the N or C terminus.

### Chemical conjugation of the synthetic peptides to BSA, fluorescently labeled BSA (FL-BSA), and biotinylated BSA

Conjugation of the synthetic peptides to rhodamine or biotin-labeled BSA molecules (Sigma Chemical Co. No. A-7906) was conducted as described before (Adam *et al.,* 1992; Broder *et al.,* 1997). BSA was labeled with rhodamine (lissamine rhodamine B sulfonyl chloride, mixed isomers, from Molecular Probes) according to the manufacturer's instructions and as described before (Karni *et al.,* 1998). Biotinylated BSA molecules were purchased from Sigma. The conjugates obtained were concentrated using a VivaScience concentrator (Sartorius), MWCO 50,000.

### Panning and screening of the phage display scFv library

Panning of the human scFv phage display library (Harrison *et al.,* 1996; Nissim *et al.,* 1994) was conducted as follows: to allow coating with the corresponding protein molecules, two immunotubes (5 ml, NUNC) were incubated overnight, in parallel, with rotation at room temperature, one with a solution containing the BSA–VprN conjugate and the other with a solution containing only

BSA, both at a concentration of 0.1 mg/ml in carbonate buffer (0.05 M $Na_2CO_3$/0.05 M $NaHCO_3$, pH 9.6). Following incubation, the tube coated with the BSA–VprN conjugates was washed three times with 1× PBS and then incubated for 2 h at 37°C with 2% (w/v) skim milk (DIFCO) in 1× PBS (2% MPBS). After an additional three washes with 1× PBS, the phage library (4 ml in 1% MPBS) was added and the tube was incubated first for 30 min with and then for 1.5 h without rotation at room temperature. The supernatant was removed, and the tube containing bound phage particles was washed 10 times first with 1× PBS–0.05% Tween 20 and then with 1× PBS. For elution of surface bound phages, a volume of 1 ml of 0.1 M triethylamine was added and following incubation for 10 min at room temperature with rotation, the triethylamine solution with the eluted phages was collected and mixed with 0.5 ml of 1 M Tris–HCl, pH 7.4. At the same time, the tube coated with the BSA molecules was washed with 1× PBS, incubated with 2% MPBS and again washed with 1× PBS as described above for the BSA–VprN-coated tube. The solution containing the eluted phages was then added to this tube and incubated first for 30 min with, and then for 1.5 h without rotation at room temperature. The supernatant was collected and used for further screenings using an ELISA assay system as described below.

### PCR for determination of the CDR3 random insert sequences and of the VH gene segments of the scFv fragments

The CDR3 random insert sequence and the VH gene segments of the scFvs were elucidated following PCR of the scFv domain. DNA from bacteria infected with the selected phage particles was subjected to several rounds of amplification with 100 pM oligonucleotide primers (5CAGGAAACAGCTATGAC3 and 5GAATTTTCTGTATGAGG3). The PCR products were purified using the High Pure PCR Product Purification Kit (from Boehringer Mannheim) according to the manufacturer's instructions. Purity and size (around 900 bp) of the products were verified by agarose gel electrophoresis. The purified PCR products were subjected to sequencing.

### Expression and purification of soluble anti-VprN scFvs

Expression of soluble scFv fragments in phage-infected bacteria (HB2151) was performed as described before (Harrison *et al.*, 1996). The expressed anti-VprN scFvs were purified using CNBr-activated Sepharose (4 Fast Flow from Pharmacia) to which BSA–VprN conjugates were covalently linked according to the manufacturer's instructions. A volume of 8–10 ml of the expressed scFv fragment preparations was mixed with 2 ml of the BSA–VprN–Sepharose beads and incubated with rotation for 1 h at 4°C. At the end of the incubation period the beads were washed with 10 ml of the following solutions: first with PBS, second with PBS–0.5 M NaCl, third with 0.2 M Glycine, pH 6, and finally with 0.2 M Glycine, pH 5. The bound scFv fragments were eluted by incubation of the washed Sepharose beads with 5 ml of 0.2 M Glycine, pH 3, for 20–30 min at 4°C with rotation. Following 5 min of centrifugation at 1000 rpm, the supernatant obtained was immediately mixed with 1 M Tris–HCl, pH 7.4, dialyzed against 0.1× PBS, and then concentrated 10× using the SpeedVac Concentrator. The purity of the scFv fragments was analyzed by SDS–PAGE (Laemmli, 1970) with Mini-PROTEAN 3 Cell (from Bio-Rad) using a 12% acrylamide gel (see Fig. 2[I]).

### Determination of ligand–NLS binding by the ELISA assay system

The various ELISA assays used in the present work were—in general—carried out as follows (for further details see the legends to the figures): MaxiSorb plates (NUNC) were incubated overnight at 4°C with 200 μl of a solution containing 25–100 μg/ml of the corresponding antigen (usually an NLS–BSA conjugate) in carbonate buffer (0.05 M $Na_2CO_3$/0.05 M $NaHCO_3$, pH 9.6). Following removal of the antigen solution, the plates were washed three times with 1× PBS and were then blocked with 200 μl of 2% BSA in 1× PBS (for scFv fragments) or 2% skim milk (DIFCO) in 1× PBS (for the peptides and phages) for 2 h at 37°C. Following three washes with 1× PBS, the appropriate ligand in a total volume of 200 μl of a blocking solution was added and the plates were incubated for another 2 h at 37°C (100 μl of phage supernatant mixed with 100 μl 2% MPBS, 25 μg/ml of scFv fragment solution in 2% BSA in 1× PBS, or 100 μg/ml of peptide solution in 2% MPBS, if not specified otherwise). The plates were rewashed three times with 1× PBS, and a volume of 200 μl of the following reagents—all dissolved in the appropriate blocking solutions—was added for detection of the antigen–ligand complexes: Avidin-POD (Roshe Diagnostics) for biotin-labeled molecules (1 U/ml), anti-M13 monoclonal antibody (Amersham Pharmacia, 1:5000), for the bound phages, and a mix of mouse anti-*myc* antibody (Sigma, 1:1000) with anti-mouse–HRP antibody (Sigma, 1:2000) for the scFv fragments. Following incubation for 1 h at 37°C, the plates were washed three times with 1× PBS and the appropriate substrate was added according to the manufacturer's instructions. Each of the binding experiments was repeated at least three times. However, the data given in the figures represent results obtained from one single experiment. Quantitative differences between repeated experiments never exceeded ±15%.

### Quantitative estimation of nuclear import

Nuclear import was quantitatively determined by the ELISA-based method using biotinylated BSA–NLS transport substrates as described before (Melchior *et al.*,

1993) and in Friedler *et al.* (1998), with the below-described modifications. The Colo-205 cells were permeabilized using 1 ml of 40 μg/ml digitonin (Fluka) per $10^7$ Colo-205 cells as follows: half of the volume of the digitonin solution was applied and the remaining volume was added gradually in portions of 100–200 μl. After each addition a sample of cells was examined by phase microscopy, and when 70–80% of the cells appeared permeabilized, the process was terminated by 50- to 100-fold dilution with cold transport buffer (Melchior *et al.,* 1993).

### Expression and purification of the Vpr–GST fusion protein

A bacteria culture (strain AN3347) carrying a plasmid bearing the Vpr–GST fusion protein (Piller *et al.,* 1996) was grown with 2× TY medium supplied with 100 μg/ml of ampicilin at 37°C with shaking (225 rpm) until it reached an optical density of 0.9 at 600 nm. Protein expression was induced by 0.1 mM IPTG, and the culture was grown overnight (about 16 h) at 28°C with shaking as above. Following centrifugation (12,000 *g,* 15 min, Sorvall), the pellet obtained was suspended in 10 mM Tris–HCl buffer (1/100–1/50 of the original volume of the culture) and subjected to six 1-min cycles of sonication. The turbid solution obtained was centrifuged as above, and the supernatant obtained was added to preswelled glutathione–agarose resin (Sigma, No. G4510) in a vol/vol ratio of about 1 ml resin/20 ml solution and incubated with rotation overnight at 4°C. The agarose resin was then intensively washed in batches with 1× PBS (50–100 ml of 1× PBS per 1 ml resin), and the bound Vpr–GST was eluted by incubation of the washed resin with 1× PBS containing 30 mM of free Glutathione and 3 mM of DTT with rotation at 4°C for 1 h. The eluted protein was concentrated by VivaScience concentrators, MWCO 30,000 (Sartorius) and stored at −20°C.

### Fluorescent labeling of the recombinant Vpr–GST protein

The Vpr–GST fusion protein which was eluted from the glutathione–agarose resin was concentrated by VivaScience concentrators (MWCO 30,000) to give a final concentration of 2.5–3.0 mg/ml, dialyzed for 2–3 h against 1× PBS at 4°C, and labeled with rhodamine–maleimide (Molecular Probes) according to the manufacturer's instructions. Following 2 h of incubation at room temperature with rotation, the labeled Vpr–GST fusion protein (FL-Vpr–GST) was purified using Sephadex G-25 fine (Pharmacia). The FL-Vpr–GST was concentrated again using VivaScience concentrators (MWCO 30,000) and stored at −20°C.

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Dossier: AIDS

# Antibodies to Tat and Vpr in the GRIV cohort: differential association with maintenance of long-term non-progression status in HIV-1 infection

Max W. Richardson [a], Jyotika Mirchandani [a], Joseph Duong [a], Sammy Grimaldo [a], Virginia Kocieda [a], Houria Hendel [b], Kamel Khalili [a], Jean-François Zagury [b], Jay Rappaport [a,*]

[a] *Center for Neurovirology and Cancer Biology, 224B BLS Building, Temple University, 1900 N. 12th Street, Philadelphia, PA 19122, USA*
[b] *Laboratoire de Biologie Cellulaire, Université Pierre et Marie Curie, Paris, France*

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**Abstract**

The HIV-1 regulatory protein Tat and the accessory protein Vpr are thought to stimulate viral replication and contribute to viral pathogenesis as extracellular proteins. Humoral immune responses to these early viral proteins may therefore be beneficial. We examined serum anti-Tat and anti-Vpr IgG by ELISA in the GRIV cohort of HIV-1 seropositive slow/non-progressors (NP) and fast-progressors (FP), and in seronegative controls. Based on information obtained during a brief follow-up period (median = 20 months), NPs were sub-grouped as those maintaining non-progression status and therefore stable (NP-S), and those showing signs of disease progression (NP-P). As the primary comparison, initial serum anti-Tat and anti-Vpr IgG (prior to follow-up) were analyzed in the NP sub-groups and in FPs. Anti-Tat IgG was significantly higher in stable NP-S compared to unstable NP-P ($P = 0.047$) and FPs ($P < 0.0005$); the predictive value of higher anti-Tat IgG for maintenance of non-progression status was 92% ($P = 0.029$). In contrast, no-difference was observed in anti-Vpr IgG between NP-S and NP-P, although both were significantly higher than FPs ($P \leq 0.001$). Serum anti-Tat IgG mapped to linear epitopes within the amino-terminus, the basic domain and the carboxy-terminal region of Tat in stable NP-S. Similar epitopes were identified in patients immunized with the Tat-toxoid in a Phase I study in Milan. High titer serum anti-Tat IgG from both GRIV and Milan cohorts cross-reacted in ELISA with Tat from diverse viral isolates, including HIV-1 subtype-E (CMU08) and SIVmac251 Tat; a correlation was observed between anti-Tat IgG titers and cross-reactivity. These results demonstrate that higher levels of serum anti-Tat IgG, but not anti-Vpr IgG, are associated with maintenance of non-progression status in HIV-1 infection. Evidence that vaccination with the Tat toxoid induces humoral immune responses to Tat similar to those observed in stable non-progressors is encouraging for vaccine strategies targeting Tat.



*Keywords:* HIV-1; GRIV; Tat; Vpr; Non-progression

## 1. Introduction

Development of a safe and effective vaccine for AIDS, even one that does not provide complete protection, is a top priority internationally. Although two large vaccine studies in humans targeting gp120 are still in progress [25], vaccines based solely on outer coat proteins do not appear to be effective. Induction of cytotoxic T-lymphocyte (CTL) responses to other more conserved viral proteins such as Gag now appear more promising [14] at least in the SHIV 89.6P model [9,54]. However, because smaller proteins contain fewer potential CTL epitopes, viral escape may be a real problem with single target vaccine strategies [2,3,8]. Similarly, vaccines based on single small viral proteins may not always induce CTL responses in genetically diverse populations. For these reasons, multi-subunit vaccines targeting several well-conserved key viral proteins may ultimately prove more effective. As a potential vaccine subunit, the early regulatory protein Tat is increasingly being incorporated in experimental vaccine studies, with mixed results in terms of outcome but clear immunogenicity [2,11,15,21,22,34,44, 55]. In addition, other non-structural proteins such as Nef and Rev are also being studied as vaccine targets [18,23]. Despite varying results using Tat, Nef and Rev as vaccine targets in SHIV and SIV models of HIV-1 infection, the

* Corresponding author.
*E-mail address:* jayrapp@astro.temple.edu (J. Rappaport).


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frequent targeting of these viral proteins by CTL responses in humans with HIV-1 infection supports their further evaluation [1]. CTL targeting of Tat also appears to be important in the SIV model for control of primary viremia [3], and viral escape from immune selective pressure on Tat has been documented [2,3]. Optimization of vaccination regimens may significantly improve the efficacy of experimental vaccines in the SHIV and SIV models [8,54]. Similarly, rational identification and incorporation of other potential viral protein targets in experimental vaccines based on their conservation, importance in the viral life cycle and apparent immunogenicity in both cellular and humoral branches of the immune system—particularly in long-term non-progressors—may speed the development of an effective vaccine for AIDS.

The HIV-1 early regulatory protein Tat [5] is essential in the viral life cycle [12], and key functional domains of Tat are well conserved [19,20]. Tat is required to stimulate efficient elongation of viral transcripts, and acts as an RNA specific viral transcription factor to recruit the cellular transcriptional machinery via its interaction with the TAR RNA element present in all nascent viral transcripts [16] and host cellular proteins such as Cyclin-T/CDK9 [35]. Interestingly, Tat appears to be expressed even prior to viral integration [63], and is capable of stimulating a variety of cellular genes as an intracellular protein [17]. The HIV-1 accessory protein Vpr is also involved in viral transcription [58] and may interact with Tat directly [51]. In addition, Vpr is present in the virion [65], and acts as an immediate early protein to induce cell cycle arrest in $G_2$ and activate the viral LTR co-operatively with the Tat protein [51]. Vpr also appears to be involved in binding of the viral pre-integration complex to the nuclear pore complex [36], and may contribute to infectivity [53]. Like Tat, Vpr appears to be fairly well conserved [52], and although it is not essential for viral replication, mutations in Vpr impact viral replication adversely [50].

In addition to its intracellular activities, Tat is secreted from infected cells (Ensoli et al., 1993; Chang et al., 1997) and is present in serum [60,64]. Extracellular Tat is thought to exert numerous deleterious effects on the host immune system, including inducing immunosuppression [60,61] (Zagury, 1997). Interestingly, the HIV-1 accessory protein Vpr also appears to be present extracellularly [28], and may similarly exert immunomodulatory effects on the host immune system, including immunosuppression [6,29]. Because Tat and Vpr are present and apparently biologically active in the extracellular milieu, humoral immune responses to these viral proteins may impact viral replication and pathogenesis, and hence slow disease progression. Although both Tat and Vpr appear to be targeted by cellular immune responses in humans [1,4,27], the humoral responses to these proteins appear to be somewhat different. A number of reports have demonstrated that antibodies to Tat are associated with non-progression to AIDS [39,40,49,66], and can block viral replication in vitro [39]. In contrast, antibodies to Vpr have not been shown to be associated with non-progression to AIDS [42,43], although antibodies to Vpr can block induction of viral replication by Vpr in vitro [28]. In order to examine and compare the association of antibodies to Tat and Vpr with slow/non-progression to AIDS, we analyzed levels of serum IgG to these viral proteins in the GRIV cohort [24,38,46,66] of HIV-1 seropositive slow/non-progressors (NP) and fast-progressors (FP). Of particular importance in the analysis, NP samples were later sub-grouped based information obtained during a brief follow-up period (median = 20 months) as those maintaining non-progression status during follow-up and therefore stable (NP-S), and those showing signs of disease progression (NP-P). Initial levels of serum IgG to Tat and Vpr (prior to follow-up) were compared between the two sub-groups, and the predictive value of antibodies to Tat and Vpr in maintenance of non-progression status was determined.

## 2. Materials and methods

### 2.1. Human serum samples

Human serum samples from HIV-1 seropositive slow/non-progressors (NP) and fast-progressors (FP) were obtained from the genetic resistance to human immunodeficiency virus (GRIV) cohort [24,38,46,66]. The GRIV cohort was established in France to identify immune responses and genetic polymorphisms associated with slow/non-progression to AIDS, and consists solely of serum and PBMC DNA samples from HIV-1 seropositive Caucasian individuals. Briefly, NPs were defined as having CD4+ T-cell counts > 500 cells/μl, despite being HIV-1 seropositive for at least 8 years without the benefit of anti-retroviral therapy (ART). Based on information obtained during a brief follow-up period (median = 20 months), NPs were sub-grouped as maintaining non-progression status and therefore stable (NP-S), and those showing signs of disease progression (NP-P); initial serum samples (prior to follow-up) were used for analysis of antibodies to Tat and Vpr in these sub-groups. FPs were defined as having CD4+ T-cell counts < 300 cells/μl within 2 years of seroconversion; some FPs received ART, including HAART regimens incorporating protease inhibitors. Control serum samples were collected from HIV-1 seronegative Caucasians with informed consent at Mt. Sinai Medical Center (New York, NY, USA), and Hahnemann Hospital of Drexel University (Philadelphia, PA, USA). Seronegative and HIV-1 seropositive serum samples from patients immunized with the Tat toxoid were obtained from a Phase I clinical trial in Milan, Italy [21,22].

### 2.2. Recombinant Tat and Vpr proteins

For analysis of anti-Tat IgG in the GRIV cohort, HIV-1 IIIB (pCV1) 86 amino-acid Tat was expressed in *E. coli* as a non-fusion protein and purified under native conditions by cation exchange chromatography, phase separation and reverse phase chromatography; the purified IIIB Tat was essen-

tially endotoxin-free by LAL assay (BioWhittaker, Walkersville, MD, USA). For reciprocal endpoint dilution and cross-reactivity ELISA, HIV-1 IIIB (pCV1) 86 amino-acid Tat, HIV-1 subtype E (CMU08) Tat, full-length S/HIV 89.6P Tat and a truncated 86 amino-acid 89.6P Tat, and SIVmac251 Tat were expressed in *E. coli* as N-terminal polyhistidine fusion proteins and purified essentially as previously described [44]. Briefly, polyhistidine Tat fusion proteins were purified initially by metal-chelate affinity chromatography under denaturing conditions. N-terminal polyhistidine fusion domains were then removed by Cyanogen Bromide (CnBr) cleavage and cleaved Tat proteins were re-folded at pH 5.0 by diafiltration. Following endotoxin removal by phase separation, cleaved Tat proteins were further purified by cation exchange chromatography, desalted by reverse phase chromatography and lyophilized by freeze-drying.

For analysis of serum anti-Vpr IgG, a cDNA encoding the 96 amino-acid HIV-1 89.6 Vpr protein was first amplified by RT–PCR from total RNA of 89.6 infected PBMC. RNA was purified using Tri-Reagent (Sigma, St. Louis, MO, USA). RT was performed with 1 μg of total RNA and oligo dT priming using a GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA). PCR was performed using the primers F1 5' cagaggatagatggaacaagc 3' and B1 5' agcagttttaggctgacttc 3' and the following conditions: a 105 s hold at 94 °C, then five cycles of 94°/37°/72° each for 30 s, followed by 30 cycles of 94°/60°/72° each for 30 s, and 7 min extension at 72°. A cDNA encoding the 89.6 *vpr* gene was then gel purified using a gel extraction quick kit (Qiagen, Chatsworth, CA, USA) and ligated directly into the pCR3.1 TA cloning vector (Invitrogen, Carlsbad, CA, USA). The 89.6 *vpr* coding sequence was then amplified by PCR using primers F2 5' ggatccgatggaacaagccccagaagac 3' and B2 5' cccaagcttctaggatttactggctccatt 3', and sub-cloned into the *Bam*H1 and *Hin*dIII sites of pRSETB (Invitrogen) for expression in *E. coli* as an N-terminal polyhistidine fusion protein. The sequences of 89.6 *vpr* clones were confirmed by automated sequencing. 89.6 *vpr* was expressed in BL21CodonPlusRIL cells (Stratagene, La Jolla, CA, USA) using the CE6 bacteriophage system to deliver T7 RNA polymerase. N-terminal polyhistidine fusion 89.6 Vpr was purified by metal chelate affinity chromatography using Talon resin (Clontech, Palo Alto, CA, USA) under denaturing conditions and used directly in ELISA.

### 2.3. Tat peptides

Overlapping IIIB (pCV1) Tat peptides were synthesized at Hahnemann Hospital of Drexel University; serines were used in place of cysteines. BH10 Tat peptides *86*–101 and *cys* (20–39) were purchased from Tecnogen/Xeptagen, SpA, Piana di Monte Verna (CE), Italy.

### 2.4. ELISA

Enzyme linked immunosorbent assay (ELISA) was performed essentially as previously described (Engvall, 1971; [44]). Briefly, Lockwell C8 Maxisorb 96-well microtiter plates (Nalge Nunc, Rochester, NY, USA) were coated for 16 h at 4 °C with 0.5 μg/well Tat, Vpr or IIIB Tat peptides in 50 μl of 50 mM $Na_2CO_3$/$NaHCO_3$ pH 9.0. Plates were then washed 6× with 300 μl of 1 × PBS/0.05% Tween-20 using an automated 96-well microtiter plate washer (Dynex, Chantilly, VA, USA); plates were washed similarly between each subsequent step. Plates were then blocked for 4 h with 250 μl of 1 × PBS/3% Immunoglobulin-free BSA (Sigma, St. Louis, MO, USA). Plates were washed again, and serum was added at a dilution of 1:500 in 50 μl of 1 × PBS/1% BSA/0.05% Tween-20 for 16 h at 4 °C while shaking gently; titers were determined similarly using a range of dilutions. Positive and negative controls were used on each plate as standards. Plates were then washed again, and secondary antibody was added in 50 μl of 1 × PBS/1% BSA/0.05% Tween-20 for 2 h at RT while shaking gently. Protein G-HRP (BioRad, Hercules, CA, USA) was used at 1:1000 dilution for anti-Tat and anti-Vpr IgG ELISA, including anti-Tat IgG titers, cross-reactivity and linear epitope peptide mapping ELISA. Each sample was run in duplicate or triplicate; results are reflective of two or more separate experiments. Each serum sample was tested in parallel on BSA coated wells; background BSA reactivity was subtracted from each sample's mean optical density in Tat, Tat peptide, or Vpr coated wells. Background subtraction was particularly important for peptide mapping. The signal to noise ratio was low for unvaccinated GRIV HIV-1 seropositive samples; without subtracting the background and using seronegative controls in parallel it would be easy to conclude erroneously that all of Tat is immunogenic given higher backgrounds in many HIV-1 seropositive GRIV samples, presumably due to higher levels of IgG overall coincident with viral infection. After background subtraction, positivity in peptide mapping ELISA was determined based on a cutoff value calculated as the mean optical density reading of seronegative controls plus three standard deviations for each peptide. For cross-reactivity ELISA, samples were first determined to be positive or negative for each Tat protein based on a cutoff value determined as the mean optical density (O.D.) of HIV-1 seronegative controls ($n = 9$) plus three standard deviations.

### 2.5. Statistical analysis

Statistical analysis was performed using SPSS 10.0 (SPSS Inc., Chicago, IL) for Windows (Microsoft, Redmond, WA, USA). ANOVA with Dunnett's T3 correction for multiple comparisons was performed to compare serum anti-Tat and anti-Vpr IgG between groups; *P*-values are reported on an adjusted scale with significance set at $\propto = 0.05$ (SPSS 10.0). As the primary comparison, differences in anti-Tat and anti-Vpr IgG were compared between the two NP sub-groups (NP-S and NP-P) and FPs by ANOVA with Dunnett's T3 correction for multiple comparisons (three groups). The two NP sub-groups together comprise all the NP samples, with the exception of four NP samples for which follow-up information was unavailable; all samples were collected prior to

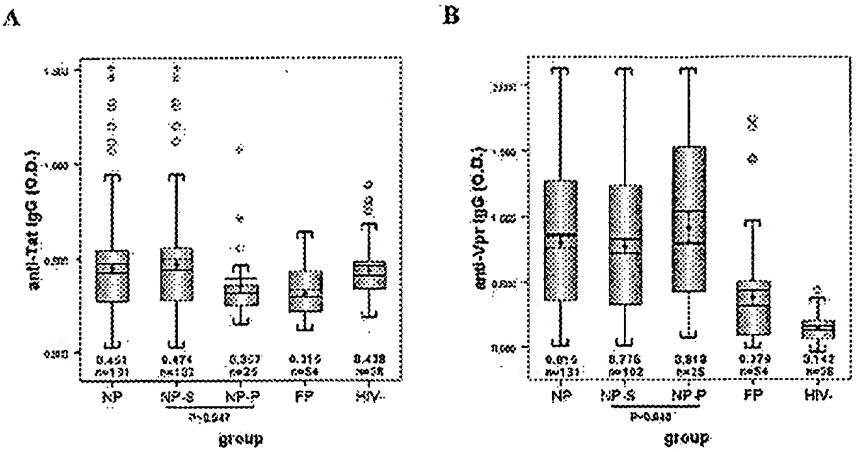


Fig. 1. Serum anti-Tat and anti-Vpr IgG levels in the HIV-1 seropositive GRIV cohort and in seronegative controls. (A) Anti-Tat IgG was significantly higher in the slow/non-progressor (NP) sub-group stable during subsequent follow-up (NP-S) compared to both NP unstable during follow-up (NP-P; $P = 0.047$) and fast-progressors (FP; $P < 0.0005$). There was no difference in anti-Tat IgG between NP-S and seronegative controls, although levels were higher in seronegatives compared to FPs ($P = 0.002$). (B) There was no difference in anti-Vpr IgG between NP-S and NP-P, although levels in both were significantly higher compared to FPs ($P < 0.0005$). Anti-Vpr IgG levels were significantly lower in seronegative controls compared to NP-S, NP-P and also FPs ($P < 0.0005$).

subsequent follow-up. Differences in distribution of NP-S and NP-P samples with high and low anti-Tat and anti-Vpr IgG were evaluated by Chi-square analysis. Correlations were assessed using Spearman's Rank test.

## 3. Results

### 3.1. Serum anti-Tat and anti-Vpr IgG

In order to determine if humoral immune responses to the viral proteins Tat and Vpr are associated with slow/non-progression to AIDS, and/or maintenance of non-progression status, ELISA was performed to evaluate serum anti-Tat and anti-Vpr IgG in GRIV slow/non-progressors (NP), non-progressor follow-up sub-groups stable (NP-S) and unstable (NP-P), fast-progressors (FP) and seronegative controls (HIV–). Comparing all five groups, serum anti-Tat IgG was significantly higher in NPs overall ($P < 0.0005$) and the stable sub-group NP-S ($P < 0.0005$) compared to FPs (Fig. 1A). Although serum anti-Tat IgG was higher in the stable NP-S sub-group compared to unstable NP-P, the difference was not significant after correcting for comparisons between five groups ($P = 0.145$). Surprisingly, serum anti-Tat IgG was also significantly higher in seronegative controls (HIV–) compared to FPs ($P = 0.003$). Similarly, anti-Tat IgG was not significantly different in seronegatives compared to NP overall or the stable NP-S and NP-P sub-groups, suggesting natural IgG recognizing domains in Tat may be present prior to infection, as has been demonstrated with IgM [49].

Examining HIV-1 seropositive samples for serum anti-Tat IgG more closely, comparison of the NP sub-groups NP-S and NP-P, and FPs (three groups) revealed that anti-Tat IgG was significantly higher in stable NP-S relative to both unstable NP-P ($P = 0.047$) and FP ($P < 0.0005$). As an additional comparison, Chi-square analysis was performed with samples from NP-S and NP-P sub-groups classified as either anti-Tat positive or negative based on a cutoff value calculated as the mean of the HIV-1 seronegative group plus two standard deviations (O.D. = 0.758). Chi-square analysis was also performed with samples from NP-S and NP-P sub-groups classified as either anti-Tat "high" or "low" based on a cutoff value calculated as the upper bound of the 95% confidence interval of the mean of the NP group overall (O.D. = 0.498). There was no difference in the distribution of anti-Tat positive samples between NP-S (13/102; 12.7%) and NP-P (1/25; 4%) by Chi-square analysis ($P = 0.211$). However, the difference in distribution of anti-Tat "high" or "low" between NP-S (35/102; 34.3%) and NP-P (3/25; 12%) was significant (Table 1; $P = 0.029$). Furthermore, the predictive value of higher initial anti-Tat IgG (prior to follow-up) in ELISA for maintenance of non-progression status during subsequent follow-up was 92% (Table 1), comparable to

Table 1
Distribution of the NP sub-groups NP-S and NP-P with high and low anti-Tat and anti-Vpr IgG

| (A) | | | |
|---|---|---|---|
| NP sub-groups | High anti-Tat IgG | Low anti-Tat IgG | Total |
| NP-S | 35* | 67 | 102 |
| NP-P | 3 | 22 | 25 |
| Total | 38 | 89 | 127 |
| **(B)** | | | |
| NP sub-groups | High anti-Vpr IgG | Low anti-Vpr IgG | Total |
| NP-S | 38** | 64 | 102 |
| NP-P | 13 | 12 | 25 |
| Total | 51 | 76 | 127 |

* Higher initial levels of anti-Tat IgG were 92% predictive of maintenance of non-progression status during subsequent follow-up; the number of NP-S with high anti-Tat IgG was significantly different than NP-P by Chi-square analysis ($P = 0.029$).

** In contrast, higher initial levels of anti-Vpr IgG were not predictive of continued non-progression during follow-up; the number of NP-S with high anti-Vpr IgG was not significantly different than NP-P.

TREC, CD4+ T-cell count, viral load and p24 antigenemia [45]; the sensitivity was 34.3%. In contrast, the predictive value of lower initial anti-Tat IgG for progression was 24.7%, also comparable to TREC, CD4+ T-cell count, viral load and p24 antigenemia. Hence, high anti-Tat IgG levels were associated with slow/non-progression to AIDS, and higher initial levels of anti-Tat IgG were predictive of maintenance of non-progression status during a subsequent follow-up period. An inverse correlation was observed in NP-S between anti-Tat IgG and p24 antigenemia ($r_s = -0.467$; $P_s < 0.0005$), as previously reported [66].

A different pattern was observed with humoral immune responses to Vpr. Comparing all five groups, serum anti-Vpr IgG was significantly higher in NPs overall ($P < 0.0005$), the stable NP-S sub-group ($P < 0.0005$) and the unstable NP-P sub-group ($P \leq 0.001$) compared to both FPs and seronegative controls (Fig. 1B). Anti-Vpr IgG was also significantly higher in FPs compared to seronegative controls ($P < 0.0005$). Examining HIV-1 seropositive samples for serum anti-Vpr IgG more closely, comparison of the NP sub-groups NP-S and NP-P, and FPs (three groups) revealed no difference between NP-S and NP-P sub-groups, unlike anti-Tat IgG, although both were higher compared to FPs ($P < 0.0005$). As an additional comparison, Chi-square analysis was performed with samples from NP-S and NP-P sub-groups classified as either anti-Vpr positive or negative based on a cutoff value calculated as the mean of the HIV-1 seronegative group plus three standard deviations (O.D. = 0.586). Chi-square analysis was also performed with samples from NP-S and NP-P sub-groups classified as either anti-Vpr "high" or "low" based on a cutoff value calculated as the upper bound of the 95% confidence interval of the mean of the NP group overall (O.D. = 1.071). There was no significant difference in the distribution of anti-Vpr positive samples between NP-S (79/102; 77.5%) and NP-P (21/25; 84%) by Chi-square analysis ($P = 0.364$). Similarly, there was also no significant difference in distribution of anti-Vpr IgG "high" or "low" between NP-S (38/102; 37.3%) and NP-P (13/25; 52%) by Chi-square (Table 1; $P = 0.178$). Although the difference was not significant, clearly a different pattern was observed with antibodies to Vpr compared to Tat. Initial anti-Vpr IgG levels appeared to be somewhat lower in NP-S compared to NP-P; fewer NP-S had high levels of anti-Vpr IgG compared to NP-P on a percentage basis. There was no correlation between serum anti-Vpr and anti-Tat IgG levels. Hence, although higher levels of anti-Vpr IgG were associated with slow/non-progression to AIDS, higher initial levels of anti-Vpr IgG were not predictive of maintenance of non-progression status during a subsequent follow-up period.

### 3.2. Linear epitope peptide mapping ELISA

In order to identify linear epitopes within Tat recognized by anti-Tat IgG, peptide mapping ELISA was performed using linear overlapping peptides derived from HIV-1 IIIB (pCV1) Tat. Linear epitopes of serum anti-Tat IgG were mapped in HIV-1 seronegative controls ($n = 7$), GRIV non-progressors stable during subsequent follow-up (NP-S; $n = 19$), unstable non-progressors (NP-P; $n = 1$), fast-progressors (FP; $n = 3$), and HIV-1 seronegative ($n = 3$) and HIV-1 seropositive ($n = 8$) Milan patients vaccinated with the Tat toxoid. GRIV samples used in peptide mapping ELISA had anti-Tat IgG against Tat protein designated as "high" in ELISA—defined as having a mean optical density above the upper 95% confidence interval of the NP group overall (O.D. = 0.498). For each sample, background subtraction of reactivity with BSA was performed prior to determining positivity. Positivity in peptide mapping ELISA was determined for each peptide based on a cutoff value calculated as the mean optical density (O.D.) of seronegative controls plus three standard deviations. Anti-Tat IgG from GRIV stable NP-S recognized linear epitopes primarily within the amino-terminus, the basic domain, and the carboxy-terminal region of Tat; several samples reacted with peptide 6.1 spanning the activation domain, but not a similar peptide "*cys*" containing cysteines (Fig. 2A). In contrast, anti-Tat IgG in fast-progressors (FP) and in a single unstable non-progressor (NP-P) mapped only to the basic domain (data not shown). Unvaccinated HIV-1 seronegative controls also recognized the basic domain of Tat to some degree, and perhaps the activation domain in a two samples (data not shown). In both seronegative and HIV-1 seropositive Milan patients vaccinated with the Tat toxoid, anti-Tat IgG recognized linear epitopes primarily within the amino-terminus and the carboxy-terminal region, although some reactivity was observed with the basic domain; one sample also reacted with peptide 6.1 spanning the activation domain, but not a similar peptide "*cys*" containing cysteines (Fig. 2B). Interestingly, in addition to being present in fewer samples, reactivity with peptides overlapping the basic domain region was much less intense compared to the amino-terminus and carboxy-terminal regions; only a small boost in reactivity to the basic domain was detected after immunization, in three samples. Unlike GRIV HIV-1 seropositive NP-S, no reactivity was detected in Milan HIV-1 seropositives with peptide "86-" spanning the extended carboxy-terminal region present in some Tat proteins; this epitope was not present in the 86 amino-acid Tat toxoid derived from IIIB (pCV1) Tat.

### 3.3. Titers of serum anti-Tat IgG

Titers of serum anti-Tat IgG were analyzed by serial endpoint dilution in ELISA. Titers were determined for GRIV NP-S ($n = 11$), NP-P ($n = 1$), and FP ($n = 2$) samples with "high" levels of anti-Tat IgG; one NP sample for whom follow-up information was unavailable was also tested. In addition, titers were determined for seronegative ($n = 3$) and HIV-1 seropositve ($n = 7$) patients vaccinated with the inactive, carboxymethylated Tat toxoid [21]. Unvaccinated seronegative controls ($n = 6$) were tested in parallel; the mean O.D. reading of seronegative controls plus three standard deviations was used as the cutoff for positivity at each dilu-

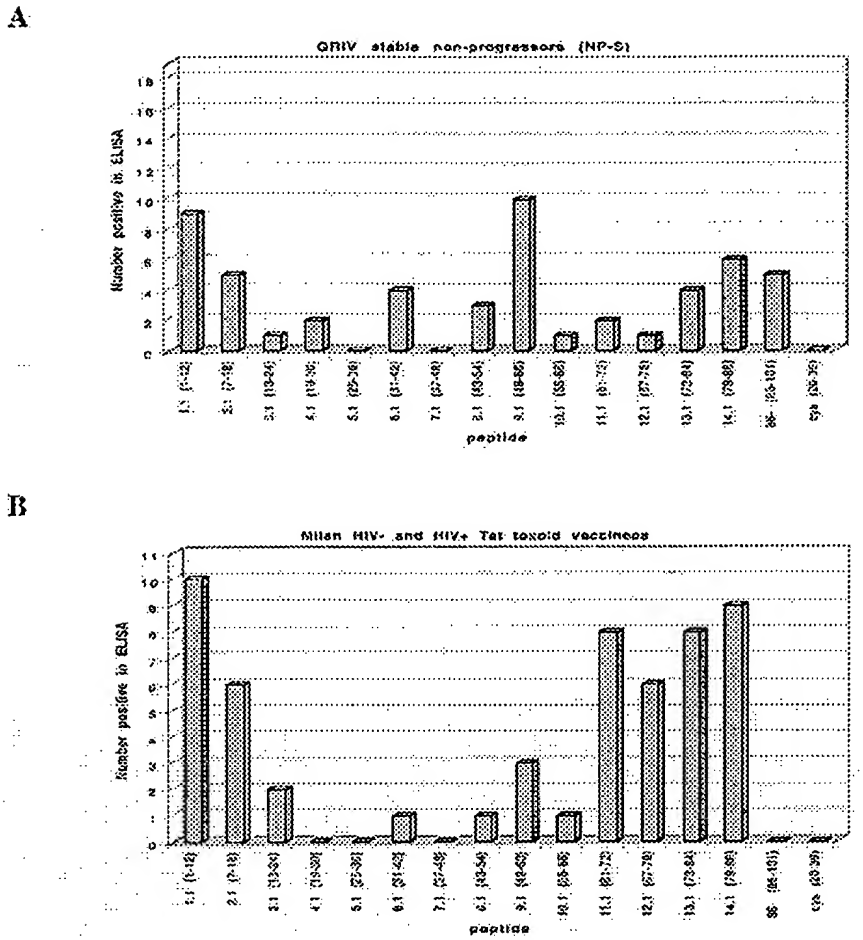


Fig. 2. Linear epitope peptide mapping ELISA of serum anti-Tat IgG. (A) Anti-Tat IgG in the GRIV HIV-1 seropositive non-progressor sub-group stable during subsequent follow-up (NP-S) maps to linear epitopes primarily within the amino-terminus, the basic domain, and the carboxy-terminal region, and possibly the activation domain. (B) Anti-Tat IgG in seronegative and HIV-1 seropositive Milan patients vaccinated with the Tat toxoid maps primarily to linear epitopes within the amino-terminus and the carboxy-terminal region, and to a lesser extent the basic domain.

tion tested. Titers ranged from 1:500 to above 1:10,000 – the highest dilution tested (data not shown).

### 3.4. Cross-reactivity of anti-Tat IgG

Because Tat is a well-conserved protein and broadly reactive immune responses to viral proteins may be beneficial, particularly for vaccine induced immunity in different geographic regions, the cross-reactivity of serum anti-Tat IgG with Tat proteins from diverse viral isolates was analyzed in ELISA. GRIV HIV-1 seropositive stable non-progressors (NP-S) and two fast-progressors (FP) with "high" levels of anti-Tat IgG were analyzed for cross-reactivity with a truncated 86 amino-acid S/HIV 89.6P Tat, full-length 102 amino-acid 89.6P Tat, HIV-1 subtype E (CMU08) Tat, and SIVmac251 Tat in ELISA (Fig. 3A). Anti-Tat IgG in a subset of GRIV NP-S and FP samples with "high" anti-Tat IgG levels cross-reacted in ELISA with the truncated 86 amino-acid 89.6P Tat, and to a lesser extent the full-length 102 amino-acid 89.6P Tat. Cross-reactivity was also apparent in several samples with HIV-1 subtype E (CMU08) Tat. One NP-S sample (136) reacted strongly with SIVmac251 Tat; this sample appears to recognize the extended carboxy-terminal region of Tat preferentially (data not shown). Cross-reactivity with the truncated 89.6P Tat was also observed in two FP samples with "high" levels of anti-Tat IgG; one sample also cross-reacted with full-length 89.6P Tat, CMU08 Tat and SIVmac251 Tat. To determine if vaccination induces broadly cross-reactive antibodies to Tat, serum from seronegative and HIV-1 seropositive Milan patients immunized with the Tat toxoid were also examined for cross-reactivity with Tat from diverse viral isolates in ELISA (Fig. 3B). Broad cross-reactivity was observed in a subset of Milan vaccines with the truncated 86 amino-acid 89.6P Tat, full-length 102 amino-acid 89.6P Tat, CMU08 Tat, and with SIVmac251 Tat. A strong correlation was observed between serum anti-Tat IgG titers and cross-reactivity in ELISA ($r_s \geq 0.508$; $P_s \leq 0.011$) in GRIV and Milan samples combined ($n = 24$). No correlation was observed between serum anti-Tat IgG titers or cross-reactivity with serum anti-Vpr IgG.

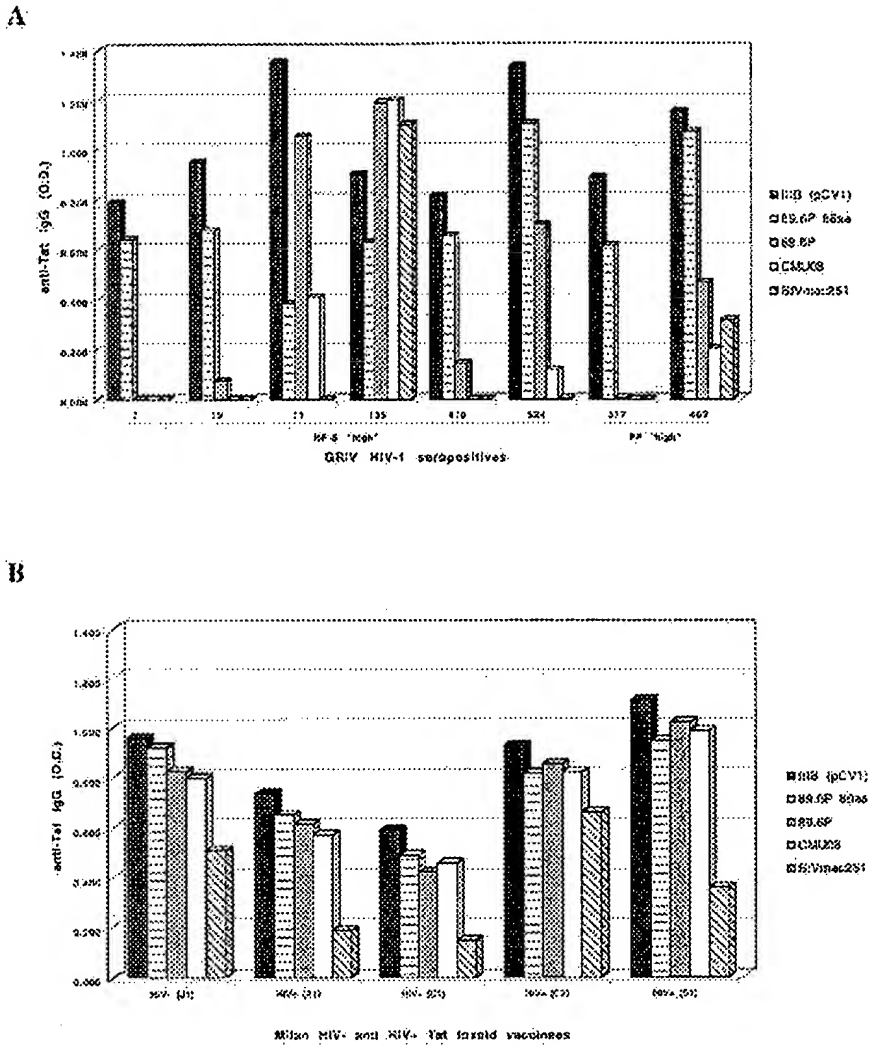


Fig. 3. High titer serum anti-Tat IgG cross-reacts with Tat from diverse viral isolates in ELISA. (A) Anti-Tat IgG in GRIV NP-S cross-reacts to varying degrees with a truncated 86 amino-acid S/HIV 89.6P Tat, full-length 89.6P Tat, HIV-1 subtype E (CMU08) Tat, and SIVmac251 Tat; cross-reactivity was also observed in two GRIV FP samples with "high" levels of anti-Tat IgG. (B) Cross-reactivity of high titer anti-Tat IgG in HIV-1 seronegative and seropositive Milan patients vaccinated with the Tat toxoid.

## 4. Discussion

Our results confirm the association of higher initial levels of anti-Tat IgG with maintenance of non-progression status during a subsequent follow-up period (median = 20 months). This association was previously demonstrated in a slightly smaller number of stable GRIV non-progressors (NP-S; $n = 78$) by stratifying the NP group overall based on "high" and "low" antibody responses to Tat, and analyzing the distribution of NP-S and NP-P samples by Chi-square analysis [66]. Furthermore, using the same dichotomized variable approach in a multivariate regression model, antibody to Tat was the most prominent predictor of disease progression, followed by viral load, antibodies to p24, Tetanus-toxoid and Nef, and p24 antigenemia. However, there was no difference in mean or median levels of anti-Tat IgG overall between unstratified NP-S and NP-P sub-groups. Here we demonstrate that there is a significant difference in anti-Tat IgG overall between unstratified NP-S and NP-P sub-groups. The difference in results between the two studies most likely reflects the use of a non-fusion Tat protein in ELISA in the current study, compared to the use of an N-terminal polyhistidine fusion protein in the previous study. The N-terminus of Tat is a key immunogenic domain in primates [44,55], and N-terminal polyhistidine fusion Tat proteins are considerably less reactive in ELISA compared to their non-fusion or cleaved Tat counterparts (data not shown). In addition, the predictive value of higher initial anti-Tat IgG levels for maintenance of non-progression status was calculated, and found to be similar to that of TREC, CD4+ T-cell count, p24 antigenemia and viral load [45].

In contrast to Tat, higher initial levels of antibodies to Vpr were not associated with maintenance of non-progression

status during subsequent follow-up. In fact, although the difference was not significant, mean levels of anti-Vpr IgG overall and both the number of samples positive for anti-Vpr IgG and the number with "high" levels of anti-Vpr IgG were somewhat higher in the unstable NP-P sub-group compared to NP-S. These results are somewhat in agreement with previous studies that found no association of antibodies to Vpr with non-progression to AIDS, although no association was found with disease progression either [42,43]. Interestingly, a separate study demonstrated a rise in serum Vpr in HIV-1 infected patients in parallel with p24 antigenemia [28], as might be expected given the presence of Vpr in the virion [65]. Hence, higher levels of anti-Vpr IgG in unstable NP-P may reflect higher viral loads and p24 antigenemia in this group compared to NP-S. These results do not preclude the potential benefit of humoral immune responses to Vpr earlier in infection; GRIV NPs overall had years of persistent infection (median = 11 years) at the time of sample collection, prior to follow-up. Indeed, although antibodies to Vpr were not predictive of maintenance of non-progression status during follow-up, higher levels were associated with slow/non-progression overall given significantly higher levels in both NPs compared to FPs. In view of evidence Vpr is targeted as frequently as Tat by CTL responses in humans [1,4], Vpr may warrant further evaluation as a vaccine component provided its potent immunosuppressive properties [6] can be removed by chemical or genetic modification.

Unlike previous studies in the GRIV [66] and other cohorts were measured in parallel with HIV-1 seronegative controls, levels of antibodies to Tat and Vpr in the GRIV cohort [42,43]. The lack of a significant difference in levels of anti-Tat IgG even in stable NP-P compared to seronegative controls supports now considerable evidence of natural antibodies to Tat [47–49], and suggests they may not be limited to IgM. It is conceivable that innate immunity to Tat may contribute to maintenance of stable non-progression status, or may prime the humoral immune response for subsequent exposure to Tat antigen. Alternatively, the highly basic nature of Tat may cause a higher background in seronegative samples due to non-specific interaction with various serum components, for example rheumatoid factor. In contrast, levels of antibodies to Vpr were significantly lower in seronegative controls even compared to fast-progressors (FP). Innate immunity to Vpr therefore does not seem to be a consideration. Of note, despite the lack of association with stable non-progression status, antibody levels to Vpr were quite high overall compared to Tat. Vpr is clearly immunogenic, perhaps in part due to its presence in the virion, although at somewhat variable copy number [56].

Because higher levels of antibodies to Tat, but not Vpr were associated with maintenance of non-progression status, linear epitope peptide mapping ELISA was performed to identify key immunogenic domains of Tat recognized by stable NP-S and associated with maintenance of non-progression status. Anti-Tat IgG mapped to linear epitopes within the amino-terminus, the basic domain and the carboxy-terminal region of Tat. Some reactivity was also seen with peptides overlapping the activation domain and containing serines in place of cysteines, but not with a peptide spanning this region and containing cysteines. Similar results were obtained with both seronegative and HIV-1 seropositive samples from individuals immunized with the Tat toxoid in a Phase I study in Milan, Italy [21,22]. We have previously observed a similar pattern of immunogenicity in rhesus macaques vaccinated with either HIV-1 IIIB or SHIV 89.6P Tat or Tat toxoid [44,55]. As seen with 89.6P versus IIIB vaccinated macaques, GRIV NP-S sample reactivity with the carboxy-terminal region of Tat seemed to extend somewhat further compared to vaccinated Milan patient samples—even in HIV-1 seropositive samples; the Milan patients were vaccinated with a Tat toxoid derived from the 86 amino-acid IIIB(pCV1) Tat, which has a truncated carboxy-terminus. Interestingly, unlike vaccinated rhesus macaques, [44,55] the Milan samples showed lower reactivity with the basic domain of Tat compared to the amino-terminus and the carboxy-terminal region, and only a small boost in reactivity to the basic domain following vaccination. This may reflect differences in vaccine preparations; clearly, the basic domain is immunogenic in humans. For example, three GRIV fast-progressor (FP) samples and one unstable NP-P with "high" antibodies to Tat mapped in parallel with NP-S and Milan samples recognized the basic domain exclusively (data not shown). It is worth noting that Tat may also contain non-linear B-cell epitopes [32], for example possibly spanning the amino and carboxyl termini [7].

Domains of Tat corresponding to linear epitopes identified in peptide mapping ELISA are associated with distinct biological activities of Tat. For example, the amino-terminus may be involved in inducing immuno-suppression through an interaction with CD26 [62]. Similarly, the basic domain of Tat facilitates its cellular uptake (Chang et al., 1997), is a strong nuclear localization signal [57], and mediates the interaction between Tat and the TAR RNA element in Tat mediated LTR transactivation [16]. The carboxy-terminal domain of Tat has been implicated in Tat mediated induction of IL-2 production [33], apoptosis [10] and is required for maximal LTR activation [26,37,59]. Antibodies recognizing these functional domains of Tat may impair Tat's biological activities as an extracellular protein, decrease viral replication and pathogenesis, and therefore contribute to maintenance of non-progression status.

Other studies have reported different results with linear epitope peptide mapping of anti-Tat IgG in HIV-1 infected humans. The entire Tat protein has been reported to be immunogenic [13,41]. However, these studies did not adjust for differences in background IgG reactivity, or use protein based blocking agents, although presumably they incorporated standard ELISA rather than high binding plates. Clearly, individuals with viral infections frequently have higher levels of IgG in serum, and hypergammaglobulinemia is a feature of HIV-1 infection in some individuals [30,31]. We observed higher levels of background reactivity in HIV-1

seropositive samples compared to seronegative controls, and therefore subtracted background reactivity of each sample (including controls) prior to scoring samples for positivity based on the mean observed in seronegatives plus three standard deviations. It is quite possible this approach is too conservative and may miss relevant epitopes. However, our results in humans are in good agreement with epitopes identified in Tat vaccinated rhesus macaques, and predicted using standard antigenicity algorithms. Furthermore, similar results were observed in GRIV NP-S samples compared to Milan samples vaccinated with the Tat toxoid—with the exception of reactivity to the extended carboxy-terminus of Tat present in the majority of primary isolates but not present in the IIIB based Milan Tat toxoid.

In order to determine how broad-based antibody responses to Tat were, we examined the ability of anti-Tat IgG in GRIV and Milan samples to cross-react with Tat from diverse viral isolates in ELISA. Several GRIV NP-S samples with "high" levels of anti-Tat IgG cross-reacted with a truncated 86 amino-acid SHIV 89.6P Tat, and to a lesser extent the full-length 102 amino-acid Tat. In addition, cross-reactivity was observed in several samples with HIV-1 subtype-E (CMU08) Tat, and in one sample with SIVmac251 Tat, demonstrating the ability of anti-Tat IgG to recognize Tat from diverse viral isolates. Cross-reactivity was also observed in two FP samples with "high" levels of anti-Tat IgG, demonstrating that recognition of a single conserved domain alone may provide broad-based responses to diverse Tat proteins. It is also possible, however, that protection in vivo may require recognition of more than one conserved domain given the differences in linear epitopes identified in stable NP-S versus unstable NP-P and FPs. Milan Tat toxoid vaccine samples also cross-reacted with diverse Tat proteins in ELISA. The larger, less homologous SIVmac251 Tat was less reactive in both GRIV and Milan samples compared to the SHIV 89.6P Tat and HIV-1 subtype E (CMU08) Tat. A strong correlation was observed with anti-Tat IgG titers and cross-reactivity with diverse Tat proteins. Similar results were observed previously with Tat vaccinated macaques [44,55].

## 5. Conclusions

Humoral immune responses to the early HIV-1 regulatory protein Tat appear to be associated with maintenance of non-progression status in HIV-1 infection. Furthermore, vaccination with the Tat toxoid induces humoral immune responses to Tat similar to those present in stable GRIV slow/non-progressors (NP-S). In contrast to Tat, humoral responses to the viral accessory protein Vpr do not appear to be associated with maintenance of non-progression status, although we cannot exclude the possibility they may be of benefit early in infection. Tat appears to be a more promising vaccine candidate at this point based on the immunogenicity of Tat in vaccine studies in rhesus macaques and humans, the association of humoral immune responses to Tat with maintenance of non-progression status in humans, and the frequent targeting of Tat by CTL responses in humans and macaques. Evidence that Tat targeted immunization in the SIV model induced immune selective pressure leading to viral escape [2] is particularly encouraging. Similarly, although the rapid decline of CD4+ T-cells appears to preclude the potential role of Tat vaccine induced immune responses in controlling viral replication in the SHIV model [44,55], the immunogenicity of Tat in both humoral and cellular branches of the immune system was impressive. Further studies in SIV models of HIV-1 infection using Tat as a component of a multi-targeted approach are underway, as are larger Phase I clinical trials of Tat toxoid alone in humans.

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# Functional Role of Residues Corresponding to Helical Domain II (Amino Acids 35 to 46) of Human Immunodeficiency Virus Type 1 Vpr

SATYA P. SINGH,[1] BRIAN TOMKOWICZ,[1] DERHSING LAI,[1] MARIA CARTAS,[1] SUNDARASAMY MAHALINGAM,[1]† VANIAMBADI S. KALYANARAMAN,[2] RAMACHANDRAN MURALI,[3] AND ALAGARSAMY SRINIVASAN[1]*

*Department of Microbiology and Immunology, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107[1]; Advanced Bioscience Laboratories, Inc., Kensington, Maryland 20895[2]; and Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104[3]*

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**Vpr, encoded by the human immunodeficiency virus type 1 genome, contains 96 amino acids and is a multifunctional protein with features which include cell cycle arrest at $G_2$, nuclear localization, participation in transport of the preintegration complex, cation channel activity, oligomerization, and interaction with cellular proteins, in addition to its incorporation into the virus particles. Recently, structural studies based on nuclear magnetic resonance and circular dichroism spectroscopy showed that Vpr contains a helix (HI)-turn-helix (HII) core at the amino terminus and an amphipathic helix (HIII) in the middle region. Though the importance of helical domains HI and HIII has been defined with respect to Vpr functions, the role of helical domain HII is not known. To address this issue, we constructed a series of mutants in which the HII domain was altered by deletion, insertion, and/or substitution mutagenesis. To enable the detection of Vpr, the sequence corresponding to the Flag epitope (DYKDDDDK) was added, in frame, to the Vpr coding sequences. Mutants, expressed through the in vitro transcription/translation system and in cells, showed an altered migration corresponding to deletions in Vpr. Substitution mutational analysis of residues in HII showed reduced stability for VprW38S-FL, VprL42G-FL, and VprH45W-FL. An assay involving cotransfection of NLΔVpr proviral DNA and a Vpr expression plasmid was employed to analyze the virion incorporation property of Vpr. Mutant Vpr containing deletions and specific substitutions (VprW38S-FL, VprL39G-FL, VprL42G-FL, VprG43P-FL, and VprI46G-FL) exhibited a negative virion incorporation phenotype. Further, mutant Vpr-FL containing deletions also failed to associate with wild-type Vpr, indicating a possible defect in the oligomerization feature of Vpr. Subcellular localization studies indicated that mutants VprΔ35-50-H-FL, VprR36W-FL, VprL39G-FL, and VprI46G-FL exhibited both cytoplasmic and nuclear localization, unlike other mutants and control Vpr-FL. While wild-type Vpr registered cell cycle arrest at $G_2$, mutant Vpr showed an intermediary effect with the exception of VprΔ35-50 and VprΔ35-50-H. These results suggest that residues in the HII domain are essential for Vpr functions.**

Members of the lentivirus family of retroviruses have been shown to contain nonstructural proteins of viral origin in addition to the structural proteins in the virus particles, a feature noted with several DNA viruses (10, 15, 21, 27). Specifically, the virus particles produced by human immunodeficiency virus type 1 (HIV-1) have been shown to contain three nonstructural proteins, designated Vif, Vpr, and Nef (6, 10, 54). A recent study, however, has questioned the specific incorporation of Vif into the virus particles (11). The virion-associated protein Vpr has been an area of intensive research with respect to understanding Vpr's role in virus infection and a potential carrier molecule to transport peptides and proteins to the assembling and mature virus particles (10, 13, 17, 22, 27, 38, 41, 44, 47, 55, 56). In addition to its ability to incorporate into virus particles (9, 10, 20, 21, 35, 45, 50), induction of apoptosis (1, 2) and differentiation (26), cell cycle arrest at $G_2$ stage (18, 30, 41, 43), nuclear localization (12, 13, 16, 28, 34, 37, 57, 58), transport of the preintegration complex to the nucleus (19, 37), transcriptional activation (8), cation-selective channel activity (40), and interaction with several candidate cellular proteins (4, 5, 14, 16, 18, 42, 49, 50, 52, 58) are some of the features of Vpr. With regard to the number of molecules of Vpr present in the virus particles, it was reported earlier that Vpr is present in amounts similar to that of Gag (7) or reverse transcriptase (22). Utilizing an epitope-tagging approach, our laboratory showed that Vpr is present in small amounts (14 to 18 molecules per virion) in the virus particles (48). Further, it was also shown that the extent of incorporation of Vpr into the virus particles can be influenced by the expression level of Vpr in cells (24).

Despite several studies, a correlation between the structure-function relationship of Vpr at the molecular level remains to be defined. Mutational analysis of Vpr, based on the secondary structure predicted by several algorithms, identified potential helical domains comprising residues 17 to 34 and 53 to 72 which are required for virion incorporation, nuclear localization, stability, and oligomerization (12, 31–35, 37, 57). Though the carboxyl-terminal region of Vpr did not have a predicted structure (residues 79 to 96), this region plays a crucial role in the cell cycle arrest function and also contributes to the sta-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Thomas Jefferson University, 1020 Locust St., JAH Rm. 461, Philadelphia, PA 19107. Phone: (215) 503-4724. Fax: (215) 923-7144. E-mail: asrinivasan@rcddil.uns.tju.edu.

† Present address: Centre for DNA Fingerprinting and Diagnostics, Hyderabad 500076, India.

bility of Vpr (10, 13). The predicted secondary structure of Vpr was also supported by circular dichroism spectroscopy studies of generated peptides corresponding to the helical domains (29). Studies by Roques and coworkers recently have provided information regarding the structure of Vpr utilizing nuclear magnetic resonance (NMR) (45, 53). It was shown that the Vpr molecule contains three helical domains, HI, HII, and HIII, involving residues 17 to 29, 35 to 46, and 53 to 78, respectively.

To address the role of helical domain HII, corresponding to the residues 35 to 46, on Vpr functions, a strategy involving deletion, insertion, and/or substitution mutagenesis was utilized. The data generated in this study indicate that HII is essential for the incorporation of Vpr into the virus particles. Further, Vpr harboring mutations in this domain failed to associate with wild-type Vpr, suggesting a role in the oligomerization function of Vpr.

## MATERIALS AND METHODS

**Cell lines.** RD, a human rhabdomyosarcoma cell line, and HeLa, a human cervix epithelioid carcinoma cell line, were obtained from the American Type Culture Collection (Manassas, Va.). Cells were maintained in Dulbecco's modified Eagle's medium (GIBCO BRL Laboratories, Grand Island, N.Y.) supplemented with 1% L-glutamine, penicillin-streptomycin, and 10% fetal bovine serum at 37°C in 5% $CO_2$.

**Construction of recombinant plasmids containing variant Vpr.** Vpr coding sequences, amplified through PCR using proviral NL4-3 DNA as a template, were cloned into the expression vector pCDNA3 (Invitrogen, Carlsbad, Calif.). The deletion, insertion, and substitution of amino acid residues in the HII domain of Vpr were carried out by using PCR methodologies (31, 47). The details of the primers used for the generation of deletion and substitution mutants are available upon request. Sequences corresponding to the Flag epitope (DYKDDDDK) were added to the 3′ end of the Vpr coding sequence to enable the detection of Vpr (46). Chimeric enhanced green fluorescent protein (EGFP)-Vpr expression plasmids were generated by fusing EGFP coding sequences at the 5′ end of the Vpr coding sequence. The integrity of plasmid DNAs was tested by application of a restriction enzyme followed by DNA sequence analysis.

**In vitro transcription/translation and RIPA of Vpr.** The coupled T7 transcription/translation system (Promega, Madison, Wis.) was used for assessing the expression of the protein directed by the Vpr clones. Incubation conditions were monitored according to the manufacturer's instructions. Radioimmunoprecipitation analysis (RIPA) of in vitro-translated proteins was carried out using polyclonal antiserum to the Flag epitope (Santa Cruz Biotechnology, Santa Cruz, Calif.) as described previously (47).

**Expression of Vpr in cells.** HeLa cells ($10^6$) in 35-mm-diameter petri dishes were infected with recombinant vaccinia virus vTF7-3 expressing T7 RNA polymerase at a multiplicity of infection of 10 for 1 h. At the end of incubation, the virus inoculum was removed and the cells were washed with phosphate-buffered saline (PBS). Vpr expression plasmids were transfected into cells using FuGENE 6 transfection reagent (Roche, Indianapolis, Ind.). Forty-eight hours after transfection the cells were washed with PBS and lysed in RIPA buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride). Cell lysate was centrifuged to remove the cell debris. Estimation of the protein content of the cell lysate was carried out using Bradford reagent (Bio-Rad, Richmond, Calif.), and 150 μg equivalent of cellular proteins was subjected to immunoblot analysis.

**Immunofluorescence assay.** HeLa cells seeded onto poly-L-lysine-coated coverslips in a 35-mm-diameter petri dish were infected with vaccinia virus vTF7-3 and were transfected with Vpr expression plasmid DNA as described above. Twenty-four hours after transfection, the cells were washed with PBS and fixed in 4% paraformaldehyde at room temperature for 30 min. After being washed three times with PBS, the cells were incubated with anti-Flag M2 monoclonal antibody-fluorescein isothiocyanate (FITC) conjugate (Sigma, St. Louis, Mo.) at 37°C in a humidified incubator for 90 min. Following several washes with PBS, the cells were incubated with 4,6-diamidino-2-phenylindole (DAPI) (0.1 μg/ml) to counterstain the nuclei, washed three times with PBS, and mounted on glass slides using a Slow Fade antifade reagent (Molecular Probes, Eugene, Oreg.). Immunofluorescence was detected using a Zeiss Axiovert 100 inverted fluorescence microscope with an attached Bio-Rad MRC 600 laser scanning confocal imaging system. To produce a merged image, each fluorochrome was recorded and the superimposed images were generated with Image-Pro software (Media Cybernetics, Silver Spring, Md.).

**Cell cycle studies.** To assess the effect of mutant Vpr on the cell cycle, we utilized a chimeric protein approach in which EGFP was fused to the amino terminus of Vpr. HeLa cells were transfected with EGFP-Vpr-encoding plasmids by the calcium phosphate precipitation method (48). At 48 h posttransfection, the cells were washed with PBS, trypsinized, diluted with PBS, and pelleted. The cells were resuspended in PBS and were gated on the fluorescence-activated cell sorter (FACScan; Coulter Apex Elite, Hialeah, Fla.) for both the EGFP-positive and -negative populations. The EGFP-positive and -negative cells were pelleted and resuspended in 80% ice-cold ethanol for 30 min. Following an additional wash with PBS, the cells were incubated in PBS containing RNase A (50 μg/ml) and propidium iodide (40 μg/ml) for 60 min at 4°C. The cellular DNA content was analyzed with a FACScan apparatus. The DNA profile was analyzed by the Multicycle AV program (Phoenix Flow System, San Diego, Calif.).

**Transfection and generation of virus particles.** HIV-1 proviral DNA (pNL4-3) was modified to disrupt the expression of Vpr by an insertion (AATT) between residues 63 and 64 within the Vpr coding region (NLΔVpr). To generate virus particles containing wild-type or mutant Vpr, NLΔVpr proviral DNA was cotransfected with the respective Vpr expression plasmid by calcium phosphate coprecipitation method into RD cells (47). Similarly, cotransfection of NL4-3, containing an intact open reading frame for Vpr, with Vpr expression plasmids was carried out to generate virus particles for assessing the association of mutant Vpr with wild-type Vpr. The virus particles released into the culture supernatant were collected 120 h after transfection. The culture supernatants were precleared for 10 min at 10,000 rpm and subsequently spun at 40,000 rpm for 3 h using sucrose density gradient centrifugation. Virus pellets were lysed in lysis buffer (62.5 mM Tris-HCl [pH 6.8], 0.2% sodium dodecyl sulfate, 1% β-mercaptoethanol, 10% glycerol), and a p24 antigen assay was used to quantitate the amount of protein present in the virus particles.

**Immunoblot analysis.** Virus samples, normalized on the basis of p24 antigen values obtained using an enzyme-linked immunosorbent assay (Organon Teknika, Durham, N.C.) were immunoprecipitated with polyclonal antiserum to Flag epitope (Santa Cruz Biotechnology) and protein A-Sepharose CL-4B (Amersham Pharmacia Biotech, Piscataway, N.J.) at 4°C overnight. The Sepharose beads were then washed and boiled in sample buffer for 5 min, and immunoprecipitated proteins were separated on NuPAGE 10% *N,N*-methylenebisacrylamide–Tris gel followed by transfer onto a nitrocellulose membrane. Membranes were blocked with 5% nonfat dry milk and incubated with rabbit polyclonal antiserum to Flag epitope for 2 h. The membranes were washed three times for 10 min each with TBST (20 mM Tris [pH 7.5], 500 mM NaCl, 0.05% Tween-20) and then probed with secondary antibody (anti-rabbit immunoglobulin AP conjugate; Promega), washed again with TBST, and developed with CDP-Star as the chemiluminescent substrate (Promega).

## RESULTS

**Structural features and generation of mutant Vpr.** The predicted secondary structure as indicated by several algorithms combined with site-specific mutagenesis studies showed that Vpr contains helical domains with a basic amino acid enriched C terminus (12, 31–35, 37, 57). Recently, Wecker and Roques (53) reported the structure of Vpr utilizing NMR spectroscopy (Fig. 1). The amino-terminal segment of Vpr comprising amino acids 1 to 51 has been shown to have three turns around the first three proline residues P5, P10, and P14. This is followed by a long helix-turn-helix motif encompassing residues 17 to 46 with another turn extending from residues 47 to 49. The helix-turn-helix motif corresponds to residues 17 to 29 (helical domain; HI), 30 to 34 (β-turn type IV), and 35 to 46 (helical domain; HII). HII is less amphipathic than HI. The studies involving the C-terminal fragment of Vpr corresponding to residues 52 to 96 showed a long amphipathic helix (residues 53 to 78; HIII) followed by a less-defined domain extending from residues 79 to 96.

With respect to the structure-function relationship of Vpr, molecular analyses involving site-specific mutagenesis have provided useful information (12, 31–35, 57). However, there is no information available regarding the role of residues present in the HII domain of Vpr. To evaluate the role of the residues in this domain, we have considered an approach involving a combination of deletion and site-specific mutagenesis. PCR-based methods were used to generate Vpr mutants lacking 1, 5, 9, and 14 residues in the HII domain and the adjoining region (Fig. 1A). In addition, a variant containing a hinge region (GGSSG) in place of the deleted residues in the HII domain was also generated. Further, to enable the detection of Vpr, sequences corresponding to the Flag epitope were fused in frame to the 3′ end of the Vpr coding sequence. Substitution

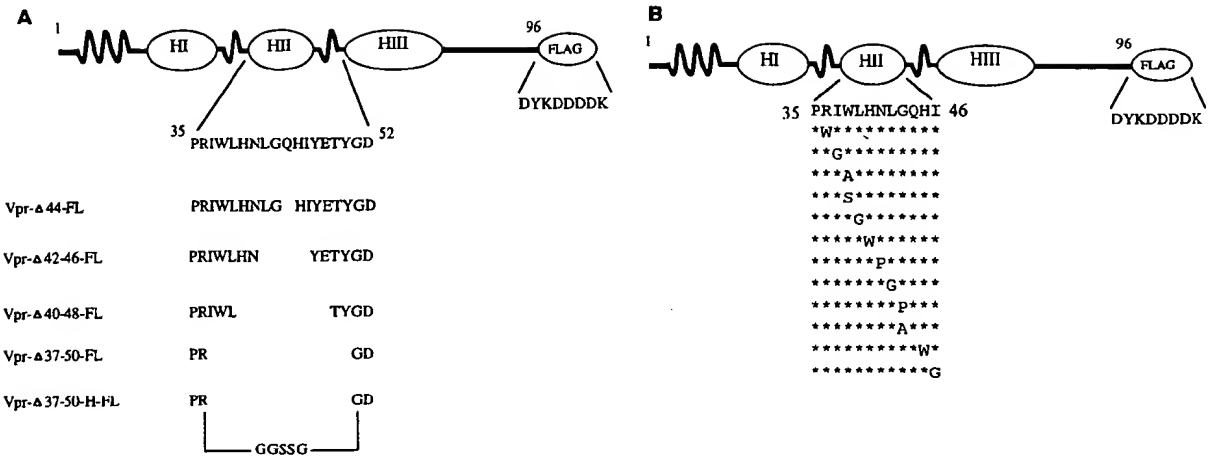


FIG. 1. Schematic representation of wild-type and mutant Vpr. The sequences corresponding to the Flag epitope were added to the 3′ end of the Vpr coding sequence. (A) The residues deleted from the HII domain and the adjoining region and the designations of mutants are indicated. (B) Substitutional mutational analysis of residues in the HII domain.

Vpr mutants (Fig. 1B) were also generated utilizing similar methods.

**Effect of mutations in helical domain II on Vpr expression.** As the Vpr expression plasmid contains the T7 promoter upstream of the coding sequences, the protein directed by each plasmid was tested using an in vitro transcription-coupled translation system (TNT; Promega). In vitro-translated proteins were immunoprecipitated with polyclonal Flag antiserum. The mutant Vpr protein was detected at the same level as the wild-type Vpr-FL protein (data not shown). As expected, the deletion of various numbers of amino acid residues (1 to 14) resulted in mutant proteins with mobilities different from that of the wild-type Vpr-FL. We also utilized recombinant vaccinia virus vTF7-3 expressing T7 polymerase to study the effect of mutations in the HII domain on the expression of Vpr in cells. vTF7-3-infected HeLa cells were transfected with wild-type or mutant Vpr expression plasmids by FuGENE 6 transfection reagent. Cell lysates, prepared 48 h after transfection, were subjected to immunoblot analysis using Flag antibodies. Transfection with each of the deletion mutants resulted in detectable levels of Vpr-FL in cell lysate (Fig. 2A). The electrophoretic mobilities of the mutant Vpr proteins were similar to those in the data for the corresponding proteins translated in vitro. Analysis of substitution mutants indicated that the protein directed by VprW38S-FL was highly unstable (Fig. 2B). In addition, mutants VprW38A-FL, VprL39G-FL, VprL42G-FL, and VprI46G-FL showed an altered stability in comparison to Vpr-FL.

**Incorporation of mutant Vpr into virus particles.** To address the role of the HII domain in the virion incorporation property of Vpr, we employed an assay system involving the cotransfection of HIV-1 proviral DNA containing a frameshift mutation in Vpr coding sequences (NLΔVpr) and the Vpr expression plasmid into cells to generate virus particles. The rationale for the assay is that Vpr, expressed in *trans*, will be incorporated into the virus particles directed by HIV-1 proviral DNA. The virus particles released into the culture medium were centrifuged and quantitated by a p24 antigen assay. The virus particles were normalized on the basis of p24 antigen values and subjected to immunoblot analysis to monitor the extent of incorporation of mutant Vpr into virus particles. The results showed that virion incorporation of Vpr deletion mutants is completely abolished (Fig. 3A). On the other hand, VprR36W-FL, VprI37G-FL, VprW38A-FL, VprH40W-FL, VprG43A-FL, and VprH45W-FL showed a positive virion incorporation phenotype. Interestingly, VprW38S-FL, VprL39G-FL, VprL42G-FL, VprG43P-FL, and VprI46G-FL exhibited a reduction in virion incorporation in comparison to Vpr-FL (Fig. 3B).

It was earlier reported (59) that the oligomerization property of Vpr may involve HI and downstream residues. This implies that residues in the HII domain may contribute to the dimerization/oligomerization feature of Vpr. To address this, we utilized an assay system in which the association of Vpr-FL mutants with wild-type Vpr was measured. This is an indirect assay for monitoring the oligomerization capabilities of Vpr in cells. Vpr mutants that lack or exhibit a low level of virion incorporation are ideal candidates for this assay. For this purpose, HIV-1 proviral DNA NL4-3 was cotransfected with either a Vpr-FL- or Vpr-FL-encoding mutant plasmid. Since the Flag epitope is present only in Vpr directed by the expression plasmid and is absent in the Vpr directed by the proviral DNA, the detection of Flag epitope-containing Vpr in the virus particles would indicate that Vpr-FL or a Vpr-FL mutant is incorporated into the virus particles by itself and/or in association with wild-type Vpr. The immunoblot analysis of virus particles generated by cotransfection of NL4-3 and Vpr-FL showed that a protein with a molecular mass of 14 kDa was detectable with Flag antibodies. On the other hand, a Vpr-FL mutant was not detectable in the virus particles, indicating that Vpr mutants failed to associate with wild-type Vpr (Fig. 4A). Since the number of molecules of Vpr present in the virus particles is low (14 to 18 molecules per virion), it is likely that

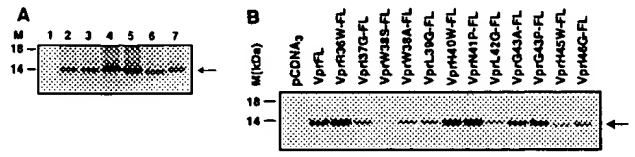


FIG. 2. Expression of wild-type and mutant Vpr. (A) Immunoblot analysis of Vpr in cells. HeLa cells were infected with vaccinia virus vTF7-3 and transfected with wild-type and mutant Vpr expression plasmids. Cell lysates were processed for immunoblot analysis as described in Materials and Methods. M, molecular mass markers (in kilodaltons). Lanes: 1, pCDNA3; 2, Vpr-FL; 3, VprΔ44-FL; 4, VprΔ42-46-FL; 5, VprΔ40-48-FL; 6, VprΔ37-50-FL; 7, VprΔ37-50-H-FL. Arrow, position of proteins. (B) Expression of Vpr harboring substitutions in HII domain in HeLa cells.

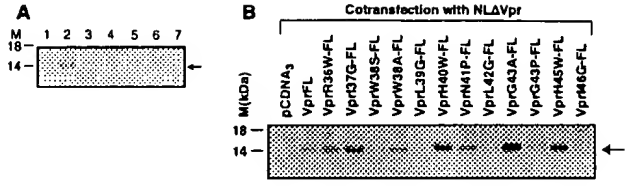


FIG. 3. Immunoblot analysis of virus particles using antibodies against Flag epitope. Viral lysates normalized on the basis of p24 antigen values were subjected to analysis following separation on Nu-PAGE 10% *N,N*-methylenebisacrylamide-Tris gel and transfer to nitrocellulose membrane. (A) Virus particles generated through cotransfection of NLΔVpr and Vpr-FL and mutant Vpr-FL expression plasmids. M, molecular mass markers (in kilodaltons). Lanes: 1, NLΔVpr; 2, NLΔVpr + Vpr-FL; 3, NLΔVpr + VprΔ44-FL; 4, NLΔVpr + VprΔ42-46-FL; 5, NLΔVpr + VprΔ40-48-FL; 6, NLΔVpr + VprΔ37-50-FL; 7, NLΔVpr + VprΔ37-50-H-FL. (B) Virion incorporation phenotypes of Vpr substitution mutants.

overexpression of mutant Vpr through a heterologous promoter may mask the wild-type Vpr incorporation expressed through HIV-1 proviral DNA. This may result in the absence of mutant Vpr-FL in the virus particles. Considering this, we also utilized a cotransfection approach in which HIV-1 proviral DNA lacking Vpr expression (NLΔVpr) and wild-type and mutant Vpr expression plasmids were transfected into cells. This was based on our earlier work showing that the expression of Vpr in *trans* leads to efficient incorporation into virus particles (392 to 550 Vpr molecules per virion). Hence, the expression of wild-type Vpr and mutant Vpr-FL in *trans* may provide an opportunity to detect mutant Vpr-FL in the virus particles through its association with wild-type Vpr. The immunoblot analysis of virus particles derived from cells cotransfected with Vpr-FL detected a band reactive to Flag antisera. However, virus particles derived from cotransfection of NLΔVpr, wild-type Vpr, and mutant Vpr-FL containing deletions did not show a band (Fig. 4B). These results suggest that mutant Vpr-FL molecules containing deletions are defective for oligomerization of Vpr. The possibility that a transdominant effect by mutant Vpr-FL on wild-type Vpr could also result in the failure to detect mutant Vpr-FL in the virus particles existed. To investigate this, virus particles derived from cotransfection were analyzed using antibodies against Vpr. Such an analysis showed similar levels of Vpr except where NLΔVpr was cotransfected with the $pCDNA_3$ vector control (data not shown), ruling out an effect on virion incorporation of wild-type Vpr.

**Subcellular localization of Vpr.** It is likely that the lack of incorporation of Vpr mutant into the virus particles may result from altered subcellular localization of the mutant protein in cells. In order to verify this, we used Vpr constructs containing the Flag epitope. Transfected HeLa cells were incubated with anti-Flag M2 monoclonal antibody-FITC conjugate followed by incubation with DAPI to stain the nucleus. As a control, we used cells transfected with the backbone pCDNA3 plasmid. As noted earlier, Vpr expressed in cells was localized to the nuclear region (Fig. 5). The observed patterns include an intense signal at the rim of the nucleus and diffuse and focal staining in the nucleus. The specificity was demonstrated by the absence of staining in the cells transfected with pCDNA3 and mock-transfected cells (data not shown). All Vpr mutants, except VprΔ37-50-H-FL, showed a localization pattern similar to that of Vpr-FL. Vpr directed by the mutant VprΔ37-50-H-FL showed an intense signal at the rim of the nucleus, and a considerable amount of protein was also present in the cytoplasm (Fig. 5). The substitution mutants designated VprR36W-FL, VprL39G-FL, and VprI46G-FL showed both nuclear and cytoplasmic localization, unlike the other substitution mutants, which localized in the nucleus (Table 1).

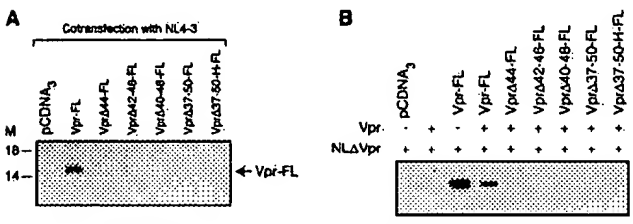


FIG. 4. Dimerization or oligomerization of Vpr in cells. (A) Extent of incorporation of mutant Vpr-FL and wild-type Vpr-FL into the virus particles directed by NL4-3 proviral DNA. Vpr was expressed in the context of HIV-1 proviral DNA. (B) Incorporation of mutant Vpr-FL in association with wild-type Vpr. Vpr was expressed through a heterologous promoter.

TABLE 1. Effect of mutations on Vpr functions[a]

| Designation of Vpr | Virion incorporation[a] | Stability[b] | Subcellular localization[c] | Oligomerization[d] |
|---|---|---|---|---|
| Vpr-FL | + | + | N | + |
| VprΔ44-FL | − | + | N | − |
| VprΔ42-46-FL | − | + | N | − |
| VprΔ40-48-FL | − | + | N | − |
| VprΔ37-50-FL | − | + | N | − |
| VprΔ37-50-H-FL | − | + | N + C | − |
| VprR36W-FL | + | + | N + C | ND |
| VprI37G-FL | + | + | N | ND |
| VprW38S-FL | − | Reduced | N (diffuse) | ND |
| VprW38A-FL | + | + | N (diffuse) | ND |
| VprL39G-FL | − | + | N + C | − |
| VprH40W-FL | + | + | N | ND |
| VprN41P-FL | +/− | + | ND | ND |
| VprL42G-FL | − | Reduced | N | − |
| VprG43A-FL | + | + | N | ND |
| VprG43P-FL | − | + | ND | − |
| VprH45W-FL | + | Reduced | N (diffuse) | ND |
| VprI46G-FL | − | + | N + C | − |

[a] Virion incorporation was carried out using cotransfection of NLΔVpr and Vpr expression plasmids.
[b] Stability of the protein was determined using recombinant vaccinia virus expressing T7 polymerase.
[c] FITC-conjugated Flag antibodies were used for subcellular localization studies.
[d] Incorporation of mutant Vpr-FL in association with wild-type Vpr was assessed in the virus particles.
[e] +, positive; −, negative; +/−, intermediate; N, nuclear; C, cytoplasmic; ND, not done.

**Effect of mutations in helical domain II on cell cycle functions of Vpr.** It was reported earlier that Vpr induces an arrest of cells at $G_2$ phase of the cell cycle (18, 30, 41, 43). Though the C terminus of Vpr containing basic amino acids has been implicated in the cell cycle arrest function (12), mutations in the amino terminus have also been shown to have an effect in this regard. This has prompted us to evaluate the effect of mutation in the HII domain on cell cycle arrest. As the addition of residues at the C terminus of Vpr may result in loss of the cell cycle arrest function (12), we have utilized a chimeric Vpr without Flag epitope at the C terminus. To visualize the cells for expression and cell cycle arrest, the EGFP coding region was fused to the 5′ end of the Vpr coding region. Cells transfected with EGFP- and EGFP-Vpr-encoding plasmids were sorted initially based on EGFP expression. The EGFP-positive and -negative cells were fixed, stained with propidium iodide, and analyzed by flow cytometry. Mock- and EGFP-transfected cells showed $G_2/G_1$ ratios of 0.17 and 0.15, respectively (Fig. 6). Cells expressing EGFP-Vpr registered cell cycle arrest with

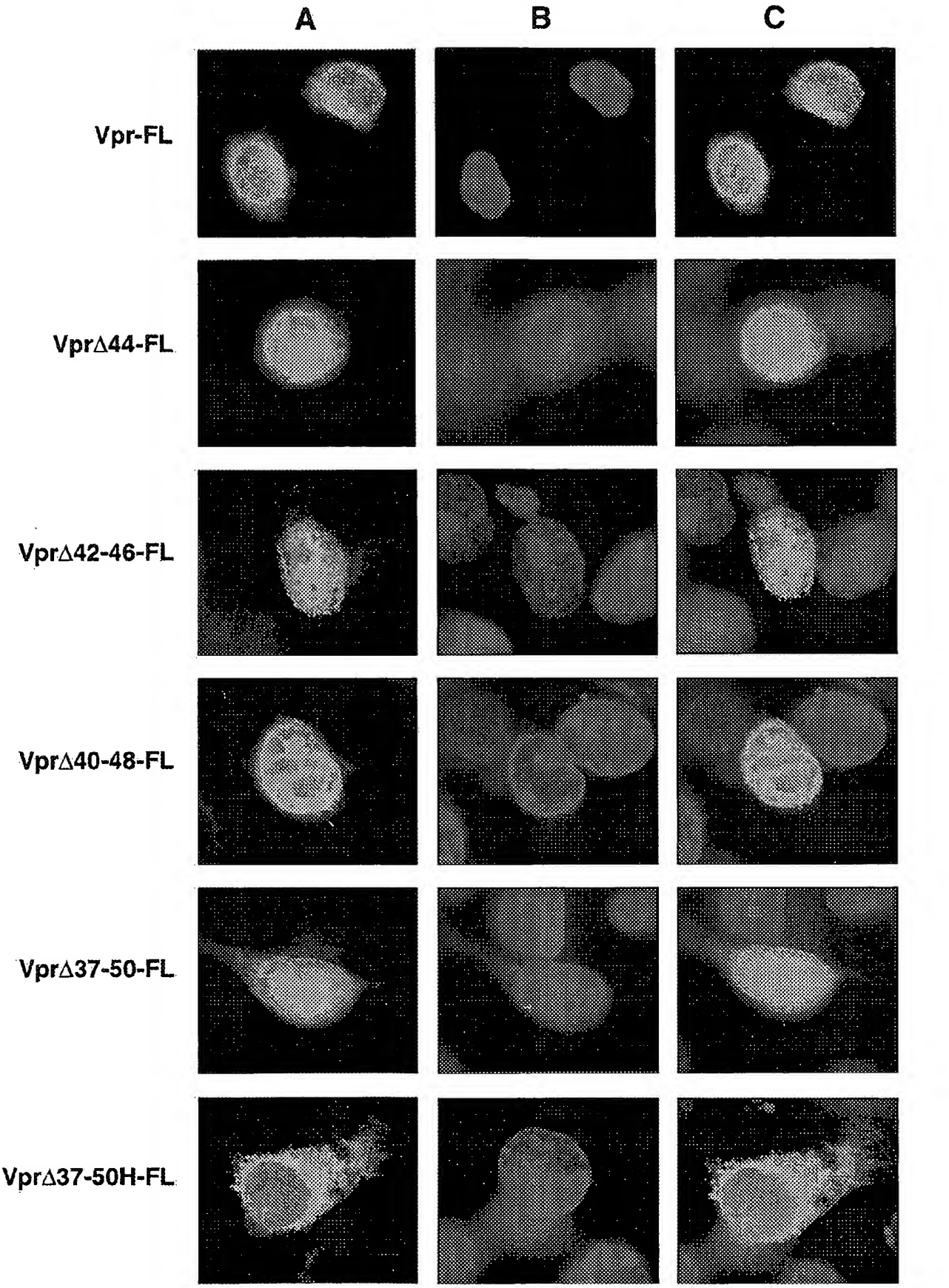


FIG. 5. Subcellular localization of wild-type and mutant Vpr. HeLa cells 24 h after transfection were fixed and stained with anti-Flag M2 monoclonal antibody-FITC conjugate followed by DAPI. Cells were analyzed using a confocal microscope at ×60 magnification. To produce a merged image, each fluorochrome was recorded and the superimposed images were generated with Image-Pro software. (A) Anti-Flag M2-FITC conjugate; (B) DAPI; (C) superimposed images.

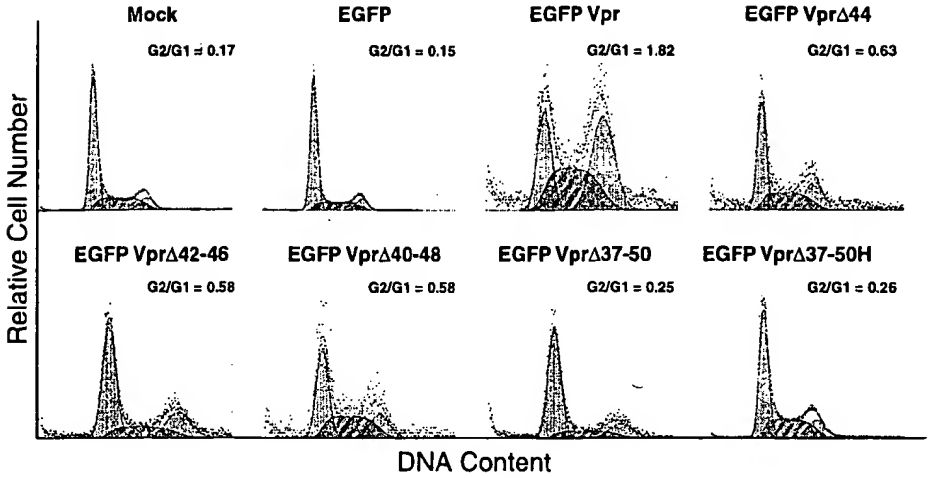


FIG. 6. Effect of wild-type and mutant Vpr on the cell cycle. HeLa cells were transfected with EGFP and EGFP-Vpr expression plasmids. At 48 h posttransfection, cells were sorted for EGFP-positive and -negative populations. The cells were stained for DNA content with propidium iodide and analyzed by flow cytometry. The $G_2/G_1$ ratio, as determined by the Multicycle AV program, of each cell population is indicated.

a $G_2/G_1$ ratio of 1.82. EGFP-VprΔ44, EGFP-VprΔ42-46, and EGFP-VprΔ40-48 mutants showed an intermediary level of cell cycle arrest with $G_2/G_1$ ratios of 0.63, 0.58, and 0.58, respectively. On the other hand, EGFP-VprΔ37-50 and EGFP-VprΔ37-50-H mutants exhibited loss of cell cycle arrest function as the $G_2/G_1$ ratios are close to the values obtained with the negative control. All the EGFP-negative cells showed nearly identical DNA profiles, similar to that of the control (data not shown).

## DISCUSSION

Vpr is a protein expressed late in HIV-1-infected cells (10, 27). Since its identification, there has been an enormous interest in understanding the functions of this protein. The demonstration that a related protein, Vpx, is present in HIV-2 and simian immunodeficiency virus virions in amounts equal to that of Gag (20) has suggested that the virion-associated proteins may have a role in the events related to virus infection analogous to the nonstructural proteins present in the virus particles directed by DNA viruses (15). The observations in support of such a role for Vpr include a positive effect on infection of macrophages by HIV-1, its role in the transport of the viral preintegration complex to the nucleus, and an effect at the level of transcription (10, 27). Furthermore, it has been shown that Vpr induces cell cycle arrest at the $G_2$ phase depending on the cell type (13) and is cytotoxic to cells through the induction of apoptosis (1, 2). With regard to the amount of Vpr, recent studies from our laboratory showed that HIV-1 Vpr is present in small amounts (14 to 18 molecules per virion) in the virus particles (48), contrary to the data reported earlier (7, 22).

In an effort to gain information about the structure-function relationship of Vpr, we have utilized the structural data that were recently reported for Vpr by Roques and coworkers (45, 53). NMR studies of the synthetic N- and C-terminal peptides comprising residues 1 to 51 and 52 to 96 of Vpr revealed a structure with three helical domains. Residues 17 to 29, 35 to 46, and 53 to 78 correspond to HI, HII, and HIII domains, respectively. Both HI and HIII domains have also been predicted by several algorithms, and mutational analyses have been carried out to determine the role of these domains in Vpr functions (32, 56, 57). In comparison to HI and HIII, the HII domain was unknown till the structural data came about, and hence there is no information available regarding the role of the HII domain in Vpr functions. The HII domain consists of 12 amino acids. The residues with hydrophilic properties, R36, N41, and Q44, are located on one side of the helix. The hydrophobic amino acids W38, L39, L42, H45, and I46 are located on the other side of the helix (53). The location of residues I37 and H40 on the former side hinders the formation of a perfect amphipathic helix (53). We have constructed several Vpr mutants involving deletion, insertion, and substitution mutagenesis approaches to understand the contribution of the HII domain to Vpr functions. The rationale for this approach is that the HII domain may be critical for maintaining the biological properties of Vpr. On the basis of this assumption, it is hypothesized that a Vpr mutant with an altered HII may not behave like a wild-type Vpr. It is likely that HII may be involved in stabilizing interactions between the HI and HIII domains or contribute to binding to Gag for its incorporation into the virus particles. The parameters that were used for assessing the effect of the mutations in the HII domain of Vpr include protein expression, stability, virion incorporation, subcellular localization, and cell cycle arrest. The results presented here indicate that the HII domain is critical for the Vpr functions. While the expression and stability of wild-type Vpr and mutants except VprW38S-FL, VprL42G-FL, and VprH45W-FL remain the same in transfected cells, the alterations in the HII domain exerted a drastic effect on the incorporation of Vpr into the virus particles. Vpr harboring mutations in the HII domain failed to get incorporated into the virus particles. A deletion of even one residue at the C terminus of the HII domain (Q44) abolished virion incorporation, similar to what was found for other deletion mutants. The deletion in VprΔ42-46-FL is confined to the HII domain comprising four residues. On the other hand, mutants VprΔ40-48-FL, VprΔ37-50-FL, and VprΔ37-50-H-FL involved deletion of residues in HII and also adjoining residues 47 to 50, which have been shown to form a Y turn. The substitution mutational analysis provided evidence supporting a crucial role for the hydrophobic residues in the virion incorporation function. Specifically, substitutions targeting W38, L39, L42, and I46 resulted in a drastic reduction in the virion incorporation of Vpr with the exception of H45. Similar studies involving the residues located on the side of the helix opposite to the hydrophobic residues (R36, I37,

H40, and N41) showed that substitution did not abrogate the virion incorporation function.

In addition to lack of virion incorporation, mutant Vpr also failed to associate with wild-type Vpr. This was arrived at by using an indirect assay in which virus particles were generated through cotransfection of either HIV-1 proviral DNA NL4-3 or NLΔVpr and Vpr expression plasmids. The presence of a Flag epitope in the mutant Vpr and its absence from Vpr encoded by NL4-3 provide the premise for analyzing mutant Vpr in the virus particles using Flag antibodies. As the mutant Vpr-FL exhibits a negative virion incorporation phenotype, the association of mutant Vpr-FL with Vpr is the only mechanism by which mutant Vpr-FL will be incorporated into the virus particles. Since wild-type Vpr was shown to be present in the virus particles derived from cotransfection by using antibodies against Vpr, it is reasonable to suggest that the absence of mutant Vpr-FL may be due to a defect at the level of dimerization or oligomerization. Recently, Schuler et al. (45) reported that a synthetic peptide corresponding to the C terminus of Vpr (residues 52 to 96) exhibited the dimerization property. However, Vpr mutants used in this study which have mutations only in HII with an intact C terminus failed to associate with wild-type Vpr. This indicates that the observation noted with the synthetic peptide (residues 52 to 96) is not applicable to full-length Vpr. The lack of incorporation of the Vpr HII domain mutant into the virus particles could result from the lack of the protein available in a sufficient amount. However, this does not seem to be the case, as equal amounts of wild-type and mutant Vpr are shown to be present in cells. Alternatively, the residues present in the HII domain may play a crucial role in terms of facilitating the interactions between Vpr and Gag (25, 44). Such a view is indeed supported by our studies, as the substitution of hydrophobic residues W38, L39, L42, and I46 resulted in a drastic reduction in the incorporation of Vpr into the virus particles. The failure to incorporate into virus particles could be due to a result of the altered subcellular localization of mutant Vpr proteins. This possibility was tested by analyzing the cellular localization of mutant Vpr in comparison to wild-type Vpr. The studies in this regard showed that most of the mutant Vprs and wild-type Vpr-FL exhibited perinuclear and diffuse staining of the nucleus. On the other hand, cells transfected with VprΔ37-50-H-FL, VprR36W-FL, VprL39G-FL, and VprI46G-FL indicated a staining pattern involving both the rim of the nucleus and cytoplasm. As cell cycle arrest is a characteristic feature of Vpr in HIV-1-infected and Vpr expression plasmid-transfected cells, Vpr mutants harboring deletions were evaluated for this function. Though the cell cycle arrest property has been attributed to the C terminus of Vpr, mutation at the amino terminus of Vpr has an effect on the cell cycle (12). While wild-type Vpr exhibited a typical cell cycle arrest at $G_2$, several mutants showed an intermediary level of cell cycle arrest. VprΔ37-50 and VprΔ37-50-H did not have any effect on the cell cycle. Overall, the experimental data described in this study point out that the hydrophobic residues of the HII domain are essential for Vpr functions.

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# Epitope-Tagging Approach to Determine the Stoichiometry of the Structural and Nonstructural Proteins in the Virus Particles: Amount of Vpr in Relation to Gag in HIV-1

Satya P. Singh,* Derhsing Lai,* Maria Cartas,* Dawn Serio,* Ramachandran Murali,† Vaniambadi S. Kalyanaraman,‡ and Alagarsamy Srinivasan*,[1]

*Department of Microbiology and Immunology, Kimmel Cancer Center, Thomas Jefferson University, 1020 Locust Street, Philadelphia, Pennsylvania 19107; †Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104; and ‡Advanced Bioscience Laboratories, Inc., 5510 Nicholson Lane, Kensington, Maryland 20895

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We used an epitope-tagging approach to determine the ratio of Gag (structural) to Vpr (nonstructural) in the virus particles directed by human immunodeficiency virus type 1. For this purpose, chimeric Gag and Vpr expression plasmids were constructed with the Flag epitope (DYKDDDDK), and the sequences corresponding to the chimeric protein were introduced into human immunodeficiency virus type 1 proviral DNA (NL4-3) to determine the ratio in the virus particles when these proteins are expressed *in cis*. In addition, NL4-3 DNA was modified to disrupt Vpr synthesis to determine the extent of incorporation of Vpr-FL when it is expressed *in trans* through a heterologous promoter. The analysis of virus particles generated by transfection of proviral DNA into RD cells indicated that (1) the ratio of Gag to Vpr in virus particles, when Vpr-FL is expressed *in cis* (in the context of proviral DNA), is in the range of 150–200:1 (14–18 molecules of Vpr per virion) and (2) the expression of Vpr-FL *in trans* showed efficient incorporation with a Gag to Vpr ratio of 5–7:1 (392–550 molecules of Vpr). These results suggest that the presence of the same epitope on different viral proteins may provide an accurate comparison of these proteins in the virus particles. 

## INTRODUCTION

Genetic analysis of the lentivirus family of retroviruses has revealed that the genomes of these viruses are complex compared with simple retroviruses (Levy, 1998). Specifically, the genome of human immunodeficiency virus type 1 (HIV-1) has been shown to code for six auxiliary proteins in addition to the Gag, Pol, and Env structural proteins (Luciw, 1996; Levy, 1998). With respect to the viral morphogenetic events, lentiviruses follow the pathway of the type C retroviruses (Hunter, 1994). Characteristically, Gag and Gag-Pol proteins are synthesized in the cytoplasm and transported to the cell membrane, and viral assembly occurs at the cell membrane of the infected cells. Although the mechanism of transport of Gag and Gag-Pol proteins to the cell membrane is not clear, the Env protein reaches the cell membrane through the secretory pathway (Hunter, 1994). This scenario suggests that the incorporation of viral proteins into the virus particles may require their interaction either in the cytoplasm or at the cell membrane (Hunter, 1994; Cohen *et al.*, 1996).

Biochemical analysis of HIV-1 particles revealed the presence of several nonstructural proteins of viral origin in the virus particles (Cullen, 1998); these include Vif, Vpr, and Nef, which are present in different amounts in the virus particles (Levy, 1998). It has been shown that both Vif and Nef exhibit nonspecific incorporation into heterologous retrovirus particles (Cullen, 1998; Levy, 1998) in addition to the particles directed by HIV-1 Gag. A recent study reported that Vif is not present in highly purified virions (Dettenhofer and Yu, 1999). On the other hand, the incorporation of Vpr into the virus particles has been shown to be specific, involving distinct domains in HIV-1 Gag (Cullen, 1998; Emerman, 1996; Levy, 1998). To explore the mechanism or mechanisms governing the incorporation of nonstructural protein or proteins into the virus particles, we have been conducting studies on Vpr. In addition to its essential role in the infection of macrophages by HIV-1 (Emerman, 1996), the characteristic features of Vpr include cell cycle arrest at the $G_2$ stage (Emerman, 1996; He *et al.*, 1995; Jowett *et al.*, 1995; Re *et al.*, 1995; Rogel *et al.*, 1995), nuclear localization (Emerman, 1996; Zhao *et al.*, 1994a), participation in the transport of the preintegration complex (Heinzinger *et al.*, 1994), cation channel activity (Piller *et al.*, 1996), and interaction with several candidate cellular proteins (Bouhamdan *et al.*, 1996; Fouchier *et al.*, 1998; Gragerov *et al.*, 1998; Refaeli *et al.*, 1995; Stark and Hay, 1998; Tung *et al.*, 1997; Zhao *et al.*, 1994a,b). Work from our laboratory and others showed the importance of putative helical domains present in Vpr for its incorporation into virus particles (Di Marzio *et al.*, 1995; Luo *et al.*, 1998; Mahalingam

[1] To whom reprint requests should be addressed at JAH Room 461. Fax: (215) 923-7144. E-mail: asrinivasan@reddi1.uns.tju.edu.

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*et al.*, 1995; Nie *et al.*, 1998; Subbramanian *et al.*, 1998; Yao *et al.*, 1995). Furthermore, evidence was also presented for the interaction of Vpr and Gag in the cells, which may be the basis for its incorporation into virus particles (Lavallee *et al.*, 1994). Regarding Gag, a role for the p6 region in the incorporation of Vpr into the virus particles has been demonstrated through deletion and the transfer of this domain to an unrelated Moloney murine leukemia virus Gag (Checroune *et al.*, 1995; Kondo *et al.*, 1995; Lu *et al.*, 1993, 1995; Paxton *et al.*, 1993).

Despite a wealth of data available on Vpr, the information regarding the amount of Vpr incorporated into the virus particles is not clear. This information may be useful for elucidating the mechanism or mechanisms involved in the incorporation of Vpr into the virus particles and for developing Vpr as an analytical and therapeutic agent. In this regard, a related protein designated Vpx (Tristem *et al.*, 1992) has been shown to be present in HIV-2 virions in equimolar quantities to that of p27 of Gag (Henderson *et al.*, 1988). To determine the amount of Vpr incorporated into HIV-1 virus particles, we used an epitope-tagging approach on Gag and Vpr involving Flag as the epitope (Chubet and Brizzard, 1996). Combining the sensitive p24 antigen assay and immunoblot analysis of virus particles using Flag antibodies, it was estimated that the ratio of Gag to Vpr was in the range of 150–200:1 when Vpr was expressed by HIV-1 proviral DNA. A much higher level of incorporation (5–7:1 for Gag to Vpr) was noted when Vpr was expressed *in trans* through an efficient heterologous promoter.

## RESULTS

### Generation of recombinant plasmids containing sequences encoding Gag-FL and Vpr-FL and analysis of epitope-tagged protein

Gag and Vpr coding sequences were derived from HIV-1 proviral DNA (NL4-3). The sequences corresponding to the Flag epitope (DYKDDDDK) were added to the 3′ end of the Vpr and Gag followed by a termination codon using the PCR method. The Gag-FL coding sequences were bounded by *Eco*RI and *Xho*I enzyme sites at the 5′ and 3′ end, respectively. Similarly, Vpr-FL coding sequences were bounded by *Hin*dIII and *Xho*I enzyme recognition sites. The Gag-FL and Vpr-FL sequences were cloned into pCDNA3 vector, which consists of cytomegalovirus immediate-early and T7 promoter upstream and bovine growth hormone poly(A)$^+$ signal sequences downstream of the coding sequences. On cloning, the integrity of the sequences was determined by DNA sequence analysis. Similar strategy was used to generate Gag-Vpr-FL in which Vpr-FL sequences were fused in frame to the 3′ end of Gag.

Because the recombinant plasmids containing Gag-FL and Vpr-FL coding sequences have the T7 promoter, an *in vitro* transcription coupled translation system was used to analyze the protein. The synthesized proteins were tested with respective antibodies. Such an analysis showed that Gag-FL protein was detected from sera from HIV-1-infected individuals and the corresponding bands were not present in the control where only vector DNA was used. The same protein was also detected by using commercially available polyclonal antibodies against the Flag epitope. Consistent with the data obtained regarding Gag-FL, antibodies against Flag epitope reacted with the Vpr-FL. Furthermore, *in vitro* translated Gag-FL and Gag-Vpr-FL proteins showed bands of similar intensities in Western blot analysis, implying equal accessibility of Flag epitope to antibodies in the context of Gag and Vpr (data not shown).

### Expression of epitope-tagged proteins (Gag-FL and Vpr-FL) in cells and incorporation of Vpr-FL into virus-like particles

For this purpose, we used recombinant vaccinia virus expressing T7 polymerase as described previously (Serio *et al.*, 1997). HeLa cells, plated at a density of $2.5 \times 10^6$ per 100-mm Petri dish, were infected with the recombinant vaccinia virus the next day for 1 h. The cells were washed, and the plasmid DNAs were transfected either alone or in combination into cells by using Lipofectin. Approximately 40–45 h posttransfection, the culture supernatant and the cells were collected separately. On lysis of the cells, an aliquot was analyzed for the expression of Gag-FL and Vpr-FL. As observed with the *in vitro* synthesized proteins, antibodies against Flag epitope detected Gag-FL and Vpr-FL (Fig. 1A). To determine the incorporation of Vpr-FL into virus-like particles, the culture supernatant was concentrated by centrifugation using the Amicon p30 column. The virus particles were lysed, and the Gag-FL and Vpr-FL were tested by immunoblot analysis. The results indicated that transfection of Gag-FL resulted in the release of viral particles and that Vpr-FL was observed in the supernatant only in association with Gag (Fig. 1B).

### Sensitivity of the epitope-tag approach

The quantification of nonstructural proteins present in the virus particles is generally carried out with the protein of interest expressed in a prokaryotic system, followed by purification of the protein for its use as a standard in the immunoblot analysis. However, such an approach for this study may still require the use of different antibodies to characterize the extent of incorporation of Vpr in relation to Gag into the virus particles. Because the affinity of the antibodies generally varies for the respective proteins, which may complicate the interpretation of the data, we decided to generate Vpr-FL and Gag-FL using Flag epitope for quantitative assays. We reasoned that the availability of a sensitive ELISA assay

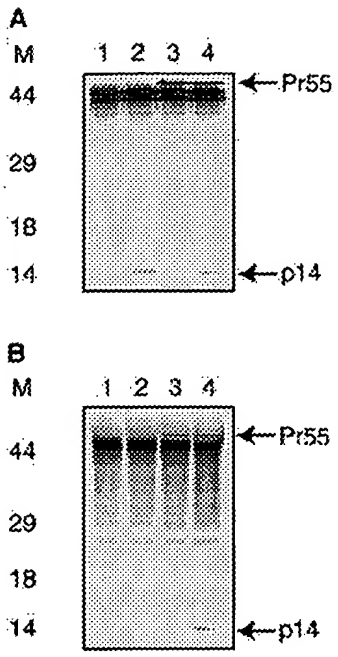


FIG. 1. Expression of Gag-FL and Vpr-FL in cells. Vaccinia virus-infected cells were transfected with the respective plasmid DNA either alone or in combination. Immunoblot analysis of the cell lysate (A) and virus-like particles (B) was carried out using Flag antibodies. M, molecular weight markers (kDa); lane 1, pCDNA3; lane 2, Vpr-FL; lane 3, Gag-FL; and lane 4, Gag-FL + Vpr-FL.

for the quantification of viral antigen in combination with immunoblot analysis using Flag antibodies may provide sufficient sensitivity to examine the ratio of Gag to Vpr in the virus particles.

The proviral DNA NL4-3 and NL-Gag-FL (Fig. 2) were independently transfected into RD cells. The virus particles released into the medium were collected at the end of 5 days posttransfection. Immunoblot analysis using antibodies against Flag epitope detected as little as 6 pg of p24 antigen equivalent of virus particles on the separation of proteins on SDS–PAGE (Figs. 3A and 3B). The extent of reactivities of Flag epitope antibodies to different concentration of proteins suggests that the approach considered here has the desired sensitivity.

### Determination of the ratio of Gag to Vpr in the virus particles

The specific incorporation of Vpr into the virus particles directed by HIV-1 is a prerequisite for determining the ratio of Gag to Vpr in the virus particles. The possibility that nonspecific association of Vpr with contaminating vesicles in the virus preparation may lead to a wrong estimation with respect to Vpr was considered. To rule this out, we used wild-type and mutant Vpr-containing substitutions in helical domain I region in cotransfection experiments with HIV-1 proviral DNA lacking Vpr expression. The immunoblot analysis of virus particles showed that incorporation was observed only for wild-type Vpr and not for mutant Vpr under the conditions used in our experiments (data not shown). To obtain information regarding the number of molecules of Vpr present in the virus particles, we decided to use an epitope-tagged Vpr in combination with and without an epitope-tagged Gag. The modified NL4-3 proviral DNA containing Vpr-FL (Fig. 2) was transfected into RD cells. The virus particles released into the medium were harvested and quantified by p24 antigen assay, and immunoblot analysis was carried out using Flag antibodies. The results (Fig. 4A) indicate that Vpr-FL can be detected in the virus particles using viral lysate. In the context of NL-Vpr-FL proviral DNA, only Vpr has the Flag epitope. In an effort to generate information regarding the ratio of Gag to Vpr in the virus particles, we used HIV-1 proviral DNA in which both the Gag and Vpr have the Flag epitope (Fig. 2). Hence, the analysis of virus particles using Flag antibodies may shed light on the relative amount of each protein present in the virus particles. The results generated with this approach may show two possible scenarios. First, an equal amount of Gag to Vpr in the virus particles would yield bands of similar intensities at Pr55 (Gag) and p14 (Vpr). Second, a differential amount of Vpr in relation to Gag would yield an intense band at Pr55 and a weak signal at p14. The immunoblot analysis of virus particles derived from transfection of NL-GV-FL revealed an altered ratio of Gag to Vpr in the range of 150–200:1 (Fig. 4B). Because Vpr-FL in HIV-1 proviral DNA is expressed as a late protein, it is likely that the level of incorporation may be due to the low level of Vpr-FL present in the cells. To test this, it was important to examine the extent of incorporation of Vpr-FL into the virus particles when Vpr-FL was expressed *in trans.* Toward this goal, cotransfection of NL-Gag-FL and Vpr-FL expression plasmid into RD cells was carried out, and the viral particles were subjected to immunoblot analysis. The results showed that Vpr-FL, expressed *in trans,* is incorporated into the virus particles at a higher level than Vpr-FL expressed in the context of the proviral DNA (Fig. 5). The ratio of Gag to Vpr was in the range of 5–7:1 in several independent experiments when only Pr55 band was considered for estimation. The inclusion of bands reactive to Flag antibodies below Pr55 showed a ratio in the range of 11–14:1.

## DISCUSSION

Among retroviruses, members of the lentivirus subfamily exhibit a unique feature of incorporation of nonstructural proteins of viral origin into virus particles similar to that of several DNA viruses (Fields *et al.,* 1996). Interestingly, HIV-1 has been shown to contain three nonstructural proteins in the virus particles: Vif, Vpr, and Nef (Cullen, 1998; Levy, 1998). The precise number of molecules present in the virus particles has been reported only for Vif (5–75 molecules) and Nef (5–20 mol-

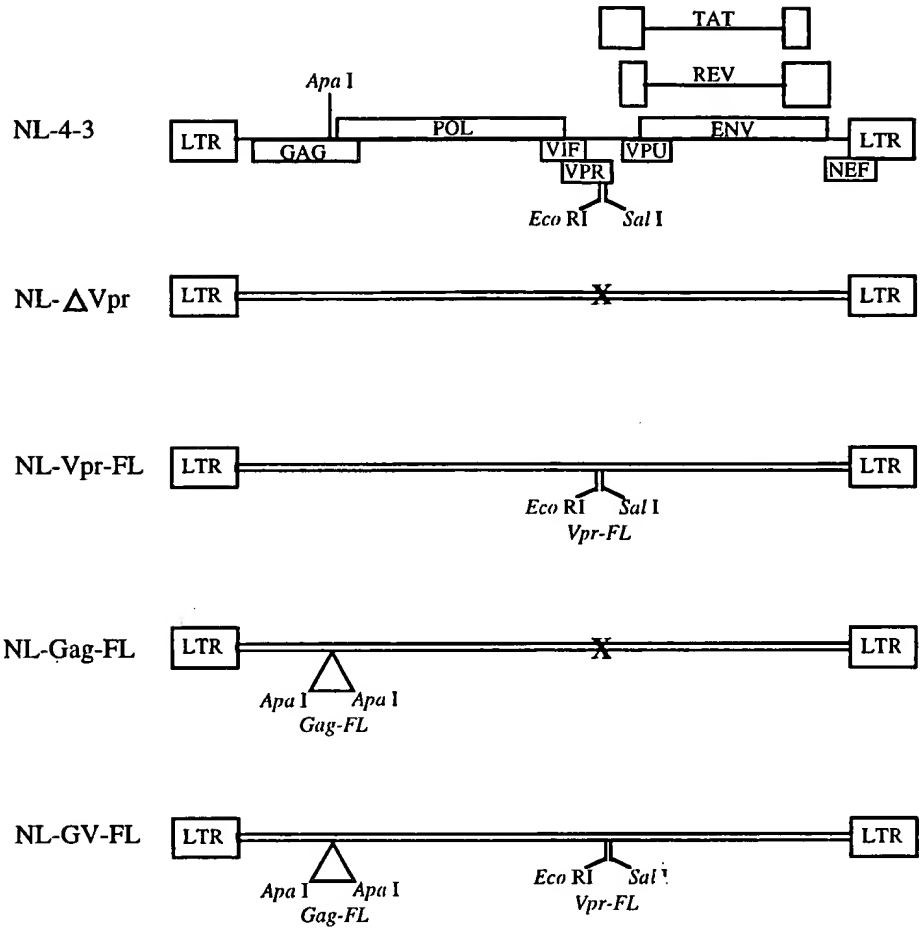


FIG. 2. Schematic representation of HIV-1 proviral DNA (NL4-3) containing Vpr, Vpr-FL, Gag-FL, and Gag-FL + Vpr-FL. The restriction enzyme cleavage sites used for cloning chimeric Vpr and Gag containing Flag epitope sequences are indicated at the top.

ecules) (Camaur and Trono, 1996; Liu *et al.*, 1995; Simon *et al.*, 1998; Welker *et al.*, 1996). The number of molecules of Vpr present in a virion is not clear. Currently, there are conflicting data indicating that the number of Vpr molecules may be similar to that of RT molecules derived from the Gag-Pol precursor protein (Kobinger *et al.*, 1998) and may also be similar to that of Gag (Cohen *et al.*, 1990). HIV-2/simian immunodeficiency virus particles contain a protein known as Vpx, which is related to Vpr (Tristem *et al.*, 1992), and studies showed that Vpx is present in the virus particles in quantities similar to that of p27 (Henderson *et al.*, 1988).

The virion-specific incorporation of HIV-1 nonstructural proteins into virus particles has led to the suggestion that these molecules can be explored to develop antiviral agents against lentiviruses, as well as analytical tools to dissect the events associated with the HIV-1 life-cycle (Fletcher *et al.*, 1997; Kobinger *et al.*, 1998; Okui *et al.*, 1998; Serio *et al.*, 1997; Wu *et al.*, 1996, 1997). To further develop this strategy, it would be desirable to know the relative number of Vpr molecules present in the virus particles. The report presented here provides an unique approach to quantify the proteins present in the virus particles. The experimental system that we used takes advantage of the addition of Flag epitope on both the Gag and Vpr. In combining the p24 antigen assay to quantify Gag and immunoblot analysis using Flag antibodies, an attempt was made to determine the ratio of Gag to Vpr in the virion. The assay that has been routinely used to determine the number of molecules of nonstructural versus structural proteins in the virus particles requires generation of the candidate protein using a bacterial expression system for use as a standard in the immunoblot analysis (Liu *et al.*, 1995). Kobinger *et al.* (1998) recently reported that the number of molecules of Vpr incorporated into the virus particles is similar to the number of RT molecules. This estimation was based on generating radiolabeled virus particles by incubating the cells with $^{35}$S-methionine. The viral proteins were subjected to immunoprecipitation by using antibodies specific to Vpr and RT and analyzed after SDS–PAGE. By taking into account the number of methionine residues present in RT and Vpr, the signal intensity of the respective protein in the autoradiogram was analyzed to estimate the number of Vpr molecules in the virus particles. These approaches assume that the affinity of the anti-

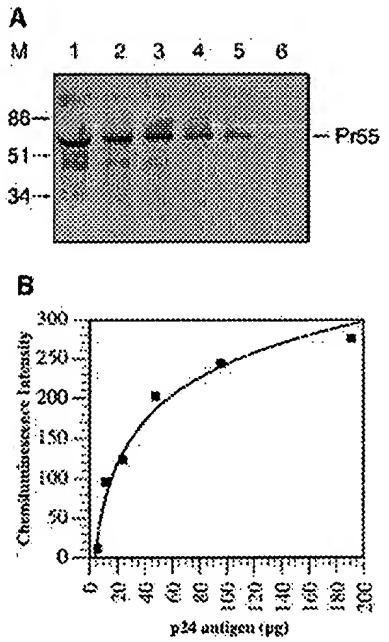


FIG. 3. (A) Immunoblot analysis of virus particles using antibodies against Flag epitope. Different concentrations of viral lysate, based on p24 antigen values, were subjected to the analysis after SDS–PAGE. M, molecular weight markers (kDa); lanes 1–6, 192, 96, 48, 24, 12, and 6pg p24 antigen equivalent of viral lysate. (B) Densitometric analysis of Gag-FL in the virus particles. Chemiluminescence intensity of each band was plotted against p24 antigen values.

bodies for the candidate proteins remains the same. The use of Flag epitope on both Gag and Vpr proteins, in our opinion, is advantageous because the same epitope on both proteins eliminates the differential affinity generally observed when using different antibodies and provides an accurate molecule-to-molecule comparison.

The Flag epitope-tagging approach was successful in obtaining the number of Vpr molecules present in the virus particles, which varies according to the mode (*cis* or *trans*) of expression of Vpr. The ratio of Gag to Vpr is found to be in the range of 150–200:1. It is assumed that there are 2750 molecules of Gag present per virion (Arthur *et al.*, 1992). Considering this, it is likely that each

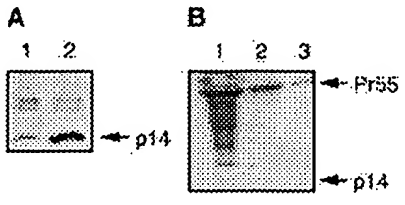


FIG. 4. Immunoblot analysis of virus particles using antibodies against Flag epitope. (A) Lane 1, NL-Vpr-FL; lane 2, virus particles derived from cotransfection of NL-ΔVpr + Vpr-FL. (B) Quantitative analysis of the ratio of Gag to Vpr in the virus particles directed by NL-GV-FL. Vpr-FL is expressed in the context of HIV-1 proviral DNA. Lanes 1–3, 50, 5, and 0.5 μl of viral lysate. Virus particles derived from NL4-3 and NL-ΔVpr did not show the corresponding Vpr-FL band.

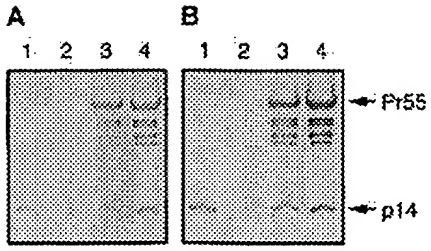


FIG. 5. Quantitative analysis of the ratio of Gag to Vpr in the virus particles when Vpr expression was directed *in trans* by a heterologous promoter. Cotransfection of NL-Gag-FL and Vpr-FL expression plasmid was carried out to derive the virus particles. On the addition of chemiluminescent substrate, the blot was exposed for 15 min (A) or 30 min (B). Lane 1, NL-ΔVpr + Vpr-FL; lane 2, NL-ΔVpr; lane 3, NL-Gag-FL + Vpr-FL (30 μl of virus lysate); and lane 4, NL-Gag-FL + Vpr-FL (50 μl of virus lysate).

virion may contain approximately 14–18 molecules of Vpr as shown by densitometric analysis. It is to be noted that the ratio 150–200:1 is only for the virus particles directed by HIV-1 proviral DNA containing both Gag-FL and Vpr-FL. As Vpr is subjected to regulation by Rev, the low incorporation of Vpr into virus particles may be due to the low amount of Vpr present in cells. Further, we also examined whether the *trans* expression of Vpr affects the efficiency of incorporation of Vpr into the virus particles. This was addressed by cotransfection of modified HIV-1 proviral DNA lacking the ability to express Vpr (NL-Gag-FL) and Vpr-FL expression plasmid in cells. The expression of Vpr *in trans* resulted in an enhancement in the incorporation of Vpr into the virus particles with 392–550 Vpr molecules per virion. The estimated number of molecules of Vpr present in the virus particles in this study is in disagreement with earlier studies (Cohen *et al.*, 1990; Kobinger *et al.*, 1998). The overestimation with regard to the number of molecules of Vpr present in the virus particles reported earlier, in our view, may stem from the methodologies used for evaluation. Because our method uses an epitope-tag approach, we have also considered the possibility that the addition of a tag may be detrimental to the virion incorporation phenotype of Vpr. Immunoblot analysis of virus particles derived from cotransfection of NLΔVpr proviral DNA with either wild-type Vpr or Vpr-FL expression plasmid showed bands with similar reactivities to antibodies against Vpr (data not shown), implying that the addition of Flag epitope did not interfere with the virion incorporation of Vpr. Furthermore, the presence of a similar amount of Vpr-FL in the virus-like particles assembled by Gag and Gag-FL also supports the above conclusion (data not shown). Hence, the results presented here suggest that one of the limiting factors for the extent of incorporation of Vpr may be the expression level of Vpr as noted for Vif (Simon *et al.*, 1998), and extrapolation of the data from the analysis of virus particles generated through expression of Vpr *in trans* as opposed to its expression *in cis* should be made with caution.

The differential level of Vpr and Gag present in the virus particles may provide clues to understanding the mechanism or mechanisms underlying the incorporation of Vpr into the virus particles. Earlier studies by Lavallee *et al.* (1994) showed an interaction between Vpr and Gag in the infected cells. It should also be noted that specific interaction of Vpr with p17 matrix (Sato *et al.*, 1996) and nucleocapsid p7 (de Rocquigny *et al.*, 1997) has been documented. It is not clear whether these interactions exist in the context of the precursor Pr55 Gag and provide help for the incorporation of Vpr into the virus particles. Because the incorporation of Vpr into virus particles can be achieved through the transfer of the carboxyl-terminal p6 domain of Gag to heterologous Gag-directed virus particles (Kondo *et al.*, 1995), it is also likely that a specific interaction between the p6 domain and Vpr is possible. The quantitative data regarding the amount of Vpr incorporation into virus-like particles directed by p55 Gag versus heterologous virus particles containing only the p6 may provide information regarding the mechanism. This quantitative study further demonstrates that Vpr can be used to develop antiviral agents targeting virus maturation and can be used as a tool to analyze the events associated with virus morphogenesis.

## MATERIALS AND METHODS

### Construction of recombinant plasmids encoding Gag-FL and Vpr-FL and cloning of sequences corresponding to epitope-tagged Gag and Vpr into HIV-1 proviral DNA

Cloning of wild-type Gag and Vpr was carried out using the pCDNA3 expression vector as described previously (Mahalingam *et al.*, 1995). For the generation of chimeric proteins containing Flag epitope, sequences corresponding to the epitope (DYKDDDDK residues) were incorporated in the 3′ primer. The primers used to generate Gag-FL were GACTGTTAAGTGTTTCAATTG [Apa(+)] located upstream of *Apa*I restriction enzyme cleavage site and CCCCCCTCGAGCTACTTGTCATCGTCGTCCTTGTAGTCTTGTGACGAGGGGTCGCT [Gag-FL(−)] corresponding to the 3′ end of Gag coding sequences followed by Flag and *Xho*I restriction enzyme cleavage site sequences. Primers TCTAGAAGCTTGCCGCCACCATGGAACAAGCCCCAGAAGAC [HKVpr(+)] corresponding to *Hin*dIII restriction enzyme cleavage site, kozak consensus, and 5′ end of Vpr coding sequences and CCCCCCTCGAGCTACTTGTCATCGTCGTCCTTGTAGTCGGATCTACTGGCTCCTT [Vpr-FL(−)] representing the 3′ end of Vpr coding sequences followed by Flag and *Xho*I restriction enzyme cleavage site sequences were used to generate Vpr-FL. For the construction of Gag-Vpr-FL, the individual PCRs were initiated with Apa(+) and TTCTGGGGCTTGTTCCATTTGTGACGAGGGGTCGCTGCC [Gag-Vpr(−)] to amplify the 3′ end of Gag and to generate Vpr-FL using the primers ATGGAACAAGCCCCAGAAGAC [Vpr(+)] and Vpr-FL(−). The DNAs derived from both reactions were purified, mixed, and amplified by using Apa(+) and VprFL(−) primers. The amplified DNA was digested with *Apa*I and *Xho*I restriction enzymes and ligated to the Gag clone cleaved appropriately.

Gag-FL and Vpr-FL sequences were introduced into HIV-1 NL4-3 proviral DNA. The schematic representation of the proviral DNAs used is shown in Fig. 2. The proviral DNA designated NLΔVpr lacks Vpr expression due to a frameshift mutation (close to the *Eco*RI recognition site) in the coding sequences. To avoid disruption of the overlap of *vpr* and *tat* at the 3′ end, the unique *Eco*RI and *Sal*I restriction endonuclease cleavage sites were used to introduce the 3′ end of chimeric Vpr from the recombinant plasmid to generate NL-Vpr-FL. Similarly, the 3′ end fragment generated by using *Apa*I from the Gag-FL plasmid was ligated to NL-ΔVpr cleaved with *Apa*I enzyme to generate NL-Gag-FL. The generation of NL-GV-FL, containing Gag-FL and Vpr-FL, used NL-Vpr-FL plasmid to introduce the Gag-FL 3′ end fragment at the *Apa*I site.

### *In vitro* transcription/translation and radioimmunoprecipitation analysis of proteins

The coupled T7 transcription/translation system (Promega, Madison, WI) was used to characterize the expression of the recombinant clones. Incubation conditions were followed according to manufacturer's instructions. Radioimmunoprecipitation analysis of *in vitro* translated proteins was carried out using polyclonal sera to Gag, Vpr, and Flag epitope (Serio *et al.*, 1997).

### Expression and incorporation of Vpr-FL into virus-like particles

To assess the expression of Vpr-FL and Gag-FL in cells and incorporation of Vpr-FL into virus-like particles, we used the vaccinia virus T7 polymerase expression system. Vpr-FL and Gag-FL expression plasmids were transfected alone and in combination into HeLa cells after infection with vaccinia virus expressing T7 polymerase. Cell lysates and culture supernatants were subjected to immunoblot analysis as described previously (Serio *et al.*, 1997).

### Generation of virus particles directed by HIV-1 proviral DNA

HIV-1 proviral DNA (wild type and modified in Gag and Vpr coding sequences) was transfected into RD cells (ATCC CCL 136, American Type Culture Collection, Rockville, MD) as described previously (Nagashunmugam *et al.*, 1992). Virus particles released into the culture medium were harvested 120 h posttransfection and quanti-

fied by p24 antigen assay with an ELISA (Organon Teknika, Durham, NC).

### Immunoblot analysis

Virus-containing culture supernatants were precleared for 10 min at 10,000 rpm and subjected to sucrose density gradient centrifugation (Serio *et al.*, 1997). Virus pellets were lysed in lysis buffer (62.5 mM Tris–HCl, pH 6.8, 0.2% SDS, 1% $\beta$-mercaptoethanol, and 10% glycerol). Samples were normalized on the basis of p24 antigen values and were run on 10% or 16% SDS–PAGE before transfer to nitrocellulose membrane. Membranes were then blocked in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) containing 5% (w/v) nonfat dry milk (Bio-Rad, Hercules, CA). Subsequent incubations with primary (anti-Flag M2; 1:1000) (Eastman Kodak Company, New Haven, CT) and secondary antisera [anti-mouse IgG (H&L) AP conjugate; 1:3000] (Promega) were performed in TTBS (TBS containing 0.05% Tween-20). Each incubation was followed by several washings in TTBS. The blot was then incubated with Western blue detection reagent, or CDP-Star (Promega) was used as the chemiluminescent substrate in a nonradioactive detection system.

### Quantification by densitometry

The intensity of the bands obtained through immunoblot analysis was used for determination of the ratio of Gag to Vpr. Blots were scanned and densitometric analysis was performed using the Molecular Dynamics ImageQuant program as described previously (Serio *et al.*, 1999).

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# Mutational Analysis of the Human Immunodeficiency Virus *vpr* Open Reading Frame

KOJI OGAWA,† RIRI SHIBATA, TAKAHIRO KIYOMASU, IKUKO HIGUCHI, YOSHIKO KISHIDA, AKINORI ISHIMOTO, AND AKIO ADACHI*

*Laboratory of Gene Analysis, Department of Viral Oncology, Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606, Japan*

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**Mutations were introduced by recombinant DNA techniques into the *vpr* open reading frame of an infectious molecular clone of human immunodeficiency virus type 1. The effect of these changes on the replicative and cytopathologic properties of the virus recovered from transfected cells was studied in several human CD4$^+$ lymphocyte cell lines. In all cases, mutant viruses were infectious and cytopathic. However, when a low-input dose was used, mutants grew significantly more slowly than the wild-type virus. The growth kinetics of *vpr* mutants were distinct from those of *vif* and *vpu* mutants.**

Human immunodeficiency virus type 1 (HIV-1) is the primary etiological retrovirus of acquired immunodeficiency syndrome and related diseases (4, 15, 18). The complete nucleotide sequences of several distinct strains of HIV-1 have revealed a very complex genetic structure (17, 20, 23, 34). In addition to the three structural genes, *gag*, *pol*, and *env*, in common with other retroviruses, six extra genes have already been identified. Of these, two *trans*-activator genes, *tat* and *rev*, are essential for virus replication (5, 7, 27, 32). *nef*, *vif*, and *vpu* are not required for virus infectivity, although mutations in these genes greatly alter the phenotypes of the virus (2, 6, 17, 26, 28, 31, 32, 34). Recently, Wong-Staal et al. reported the presence of another gene, *vpr*, which could encode a protein recognizable by sera of some HIV-1-seropositive people (37). Functional analysis of *vpr*, however, has not yet been carried out. Therefore, we investigated the effects of mutations in *vpr* on the replication and cytopathogenicity of the virus.

Figure 1 shows the structure of the genomes used for this analysis, including plasmids pNL-Nd and pNL-Ss, which are *vif* and *vpu* mutants, respectively. An infectious proviral DNA, pNL432, which expressed all known HIV-1 proteins (1, 2, 30, 31, 36), was used to generate all mutants. The mutants were constructed by insertional frameshift mutations at the restriction endonuclease sites shown in Fig. 1.

The nucleotide sequence of the *vpr* region of pNL432 was determined to compare it with the published data (Fig. 2A). pNL432 contains *vpr*, which can encode 102 codons (96 from the first methionine codon). It overlaps with the *vif* gene and terminates after the first *tat* coding exon. The deduced amino acid sequence of *vpr* is highly conserved among seven proviruses used for comparison (Fig. 2B). Of note is the presence of an infectious clone which can encode only 78 amino acids. This short version of *vpr* is due to a frameshift in the 3′ portion of *vpr* of clone HXB-2. This frameshift is observed in other clones derived from the HIV-IIIB cell line (37). *vpr* mutants in this communication all have a short amino acid sequence relative to the wild-type clone pNL432. pNL-Af2 contains 27 amino acids, and pNL-Ec and pNL-Sl contain 79.

The effects of alterations in the virus genome on the production of virus particles were determined in transfection assays. The mutants and pNL432 were introduced into SW480 cells, which efficiently express transfected DNA and produce high reverse transcriptase (RT) in the culture fluids within 24 h (1). The amount of particles produced by the mutants was comparable to that produced by wild-type DNA following transfection into SW480 cells, as determined by RT activity (Fig. 1). However, the results obtained with mutants of other regulator genes were quite different. *tat* and *rev* mutants showed no RT activity, while *nef* mutants produced higher RT activity than the wild type (not shown).

The effects of virus infection were first monitored in the Molt-4 clone 8 cell line (M4-8) by measurements of viable cell number and cell-free RT activity. This cell line was shown to be highly sensitive to cytopathic effects (CPE) caused by HIV (14). Cell-free samples of *vpr* mutants and wild-type virus particles were prepared from the supernatants of SW480 cells transfected with pNL-Af2, pNL-Ec, pNL-Sl, or pNL432 by low-speed centrifugation and filtration through a 0.22-μm filter. M4-8 cells were infected with equivalent amounts (RT units) of each virus preparation, and HIV replication was monitored (Fig. 3). M4-8 cells infected with wild-type virus displayed strong CPE, including ballooning, multinuclear giant cells, and ghost cells as early as day 7. This profound CPE was followed by a reduction in the number of viable cells (Fig. 3A). The viable cell number reached a peak at day 7 and fell gradually during the observation period. RT activity in the culture fluids began to increase on day 7 and was maximum on day 16 (Fig. 3B). The kinetics of *vpr* mutant viruses in M4-8 cell were quite different. The appearance of CPE, reduction of cell number, and RT production were observed with delayed kinetics, occurring about 7 to 10 days after confirmation of wild-type virus infection. No significant difference of kinetics was found among cells infected with three mutants. Interestingly, when 10-fold-higher input multiplicities were used, infection

* Corresponding author.
† Present address: Research Institute, Ono Pharmaceutical Co., Ltd., Osaka 618, Japan.

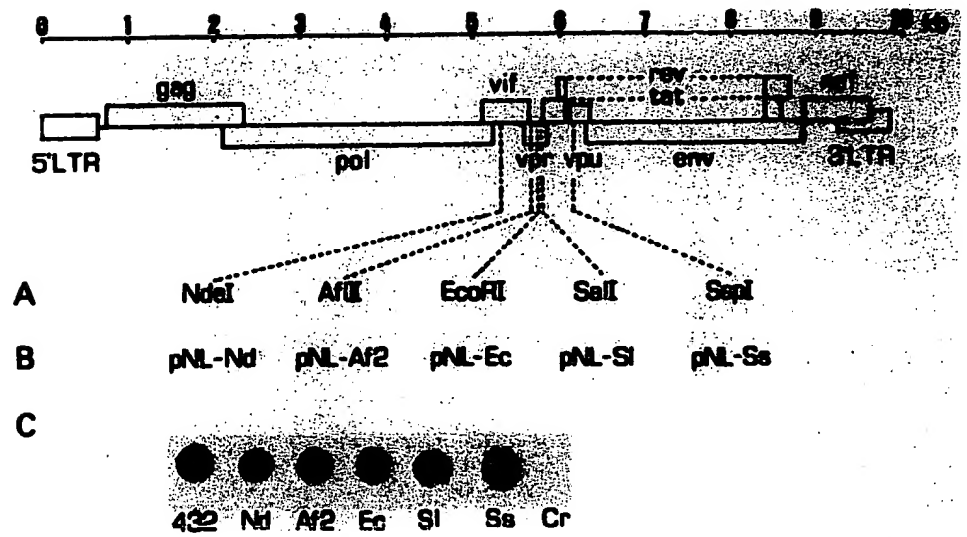


FIG. 1. Mutants used in this study. Schematic representation of HIV-1 proviral genome at the top. (A) Restriction enzyme sites used to generate mutants; (B) mutant designations; (C) transient expression of RT activity in transfected SW480 cells (ATCC CCL228). All mutants except pNL-Ss were constructed by cleaving plasmid DNA with the enzyme indicated, blunt ended by T4 DNA polymerase, and recealed by T4 DNA ligase. Thus, pNL-Nd has a 2-base-pair insertion; pNL-Af2, pNL-Ec, and pNL-Sl have 4-base-pair insertions. pNL-Ss was generated by digesting DNA with *Ssp*I, inserting an 8-base-pair *Cla*I linker by T4 DNA ligase, and reclosing by T4 DNA ligase. When necessary, the appropriate DNA fragment was first subcloned into pUC19, the mutation was introduced, and the mutated DNA was put back into the infectious clone, pNL432. DNA structure was confirmed by restriction mappings and partial sequencings. For transient transfection assays, proviral DNA was introduced into SW480 cells by the calcium phosphate coprecipitation method (11, 35). Cr in part C shows nondetectable RT activity in pUC19-transfected cells. RT activity was measured as reported previously (36). kb, Kilobases.

kinetics were indistinguishable between wild-type virus and *vpr* mutants (not shown).

We also examined cell line variation in *vpr* mutant responsiveness, using other human $CD4^+$ leukemia cell lines infected with wild-type and mutant viruses. Infection experiments were carried out as above, and CPE and RT activity were monitored at intervals (Fig. 4). In two cell lines, Molt-3 (JCRB9048) and TALL-1 (JCRB0086), similar delayed kinetics of the mutant virus infection were noted. In A3.01 cells (8), however, the time course of CPE and RT production was similar between wild-type- and mutant virus-infected cells, although RT activity persisted longer in cells infected with the mutant virus. This "tailing" of RT production in *vpr* mutant-infected A3.01 cells was always seen in several independent experiments. A3.01 cells differed from the other two cell lines in that they could produce large amounts of virus shortly after infection (Fig. 4). This property of A3.01 cells was considered to result in high multiplicities of infection at a very early stage in the time course experiment. Therefore, A3.01 cells were infected with two different doses of virus and RT activity was monitored (Fig. 5). Virus growth kinetics differed little among the three viruses when high multiplicities were used (Fig. 5, bottom). A 10-fold reduction of input dose clearly delayed the infection kinetics, particularly in *vpr* mutant-infected cells. The effect of multiplicities was not as drastic on the time course of RT production in wild type virus-infected cells. By contrast, in mutant-infected cells, the peak day of RT activity was delayed several days and the level of activity was low. Progeny viruses, produced in these four cell lines after inoculation with the mutant virus (pNL-Af2), behaved exactly like the *vpr* mutant, suggesting that "reversion" had not occurred (not shown).

The phenotype of the *vpr* mutant was compared with those of *vif* and *vpu* mutants. Published reports have demonstrated that *vif* is required for efficient virus transmission (6, 30) and *vpu* is required for virus maturation or assembly (31). A3.01 cells were infected with various viruses with quite high multiplicities. Figure 6 shows the virus growth curves of *vif*, *vpr*, *vpu*, and wild-type virus as determined by RT assays. Again, little difference was seen between wild-type and *vpr* mutant viruses. The *vif* mutant, pNL-Nd, grew so poorly that faint RT activity was detected as late as day 23 (Fig. 6A). RT activity was detected even 60 days after infection in *vif* mutant-infected A3.01 cells with very weak CPE, whereas cells infected with *vpr* mutant or wild-type virus did not produce RT activity 40 days after infection (A. Adachi and K. Ogawa, unpublished observation). Figure 6B gives a phenotypical comparison of *vpr* and *vpu* mutants. The growth curve of the *vpu* mutant was striking, although the kinetics was similar to that of the wild-type virus. A several-fold reduction in progeny virions (as determined by RT assays) relative to wild-type virus was observed, as reported (31) after the infection of A3.01 cells with a *vpu* mutant, in contrast to the phenotype of the *vpr* mutant. This growth characteristic of the *vpu* mutant was seen in another infection experiment that used 10-fold-lower input multiplicities (not shown).

Our results suggest that *vpr* is necessary for efficient replication of the virus and concurrent CPE in $CD4^+$ cell cultures. In particular, pNL-Af2, which was constructed to eliminate expression of most of the *vpr* open reading frame, is clearly inefficient for virus growth. The three *vpr* mutants, pNL-Af2, pNL-Ec, and pNL-Sl, showed similar delayed kinetics of infection relative to wild-type virus. This suggests that the C-terminal portion of *vpr* is important for *vpr* function (Fig. 2B). However, *vpr* is dispensable for growth of the virus in $CD4^+$ cells. In our assay system, the function of *vpr* is only evident after multiple rounds of productive infection have occurred, since the defective phenotype of

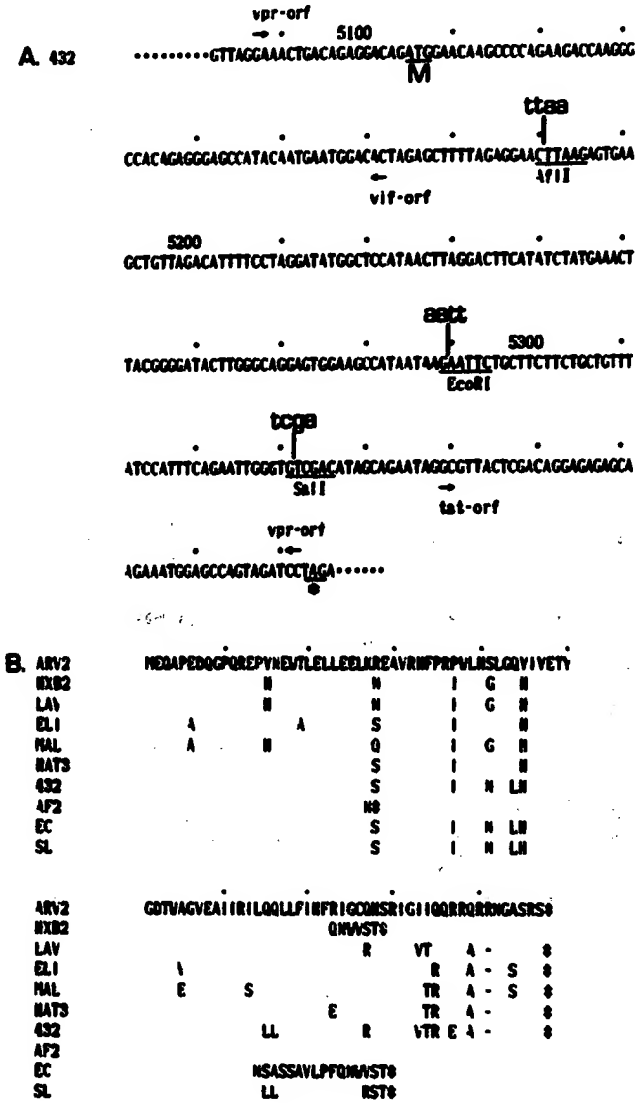


FIG. 2. Sequence of pNL432 *vpr* region. (A) Nucleotide sequence of the *vpr* region. The nucleotide numbering system of Wain-Hobson et al. (34) was used. Sequence was determined by the dideoxy sequencing method (24). Both strands were sequenced. The positions of the first methionine (M) and the stop codon (asterisk) in *vpr* are indicated. A 4-base-pair insertion is shown above the enzyme cleavage site (position also indicated). (B) Deduced amino acid sequences of *vpr* of several HIV-1 strains, ARV2 (23), HXB2 (19), LAV (34), ELI (3), MAL (3), and HAT3 (29), are shown for comparison. A one-letter amino acid code was used. Sequence is from the first methionine codon (top left) to the stop codon (asterisk, bottom right). Blank represents an amino acid identical to that of the ARV2 strain. –, Deletion of the amino acid.

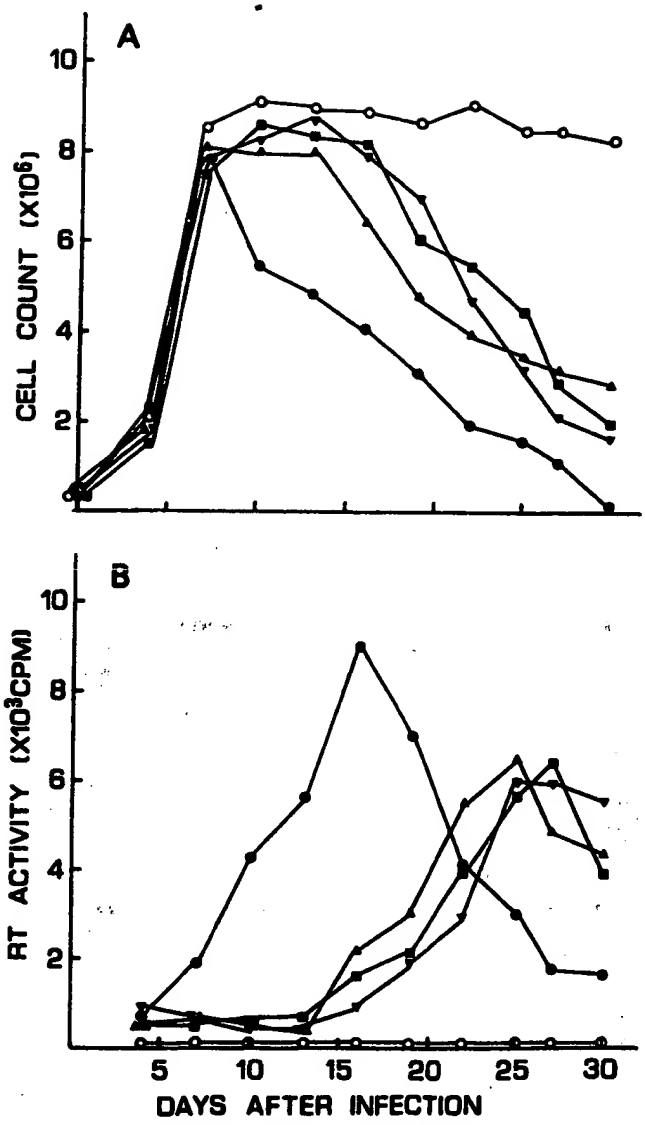


FIG. 3. Growth kinetics of *vpr* mutants (Af2, Ec, and Sl) and wild-type virus (432) in M4-8 cells. (A) Viable cell counts per culture as determined by trypan blue exclusion. (B) RT activity in culture fluids (1.5 μl of supernatants). Cells were infected with the virus in the presence of Polybrene, as reported previously (8). Equivalent amounts of virus ($2 \times 10^5$ cpm) were used to initiate infection. Symbols: ○, mock; ●, pNL432; ■, pNL-Af2; ▲, pNL-Ec; ▼, pNL-Sl.

*vpr* mutants is very dependent on input multiplicities. This mild effect of *vpr* mutation on virus replication is puzzling, since this reading frame is well conserved not only among many strains of HIV but also in the distantly related ungulate lentivirus, visna virus (28). Moreover, some sera of infected individuals recognize the bacterially expressed *vpr* products (37). Conservation of the *vpr* open reading frame suggests that it plays an important role in the life cycle of the virus. Two possible functions for *vpr* can be considered. *vpr* may be essential for the growth of HIV in cell types other than the $CD4^+$ lymphocytes used in this report. Productive infection of monocytes and macrophages has been described recently (10, 22). HIV has also been reported to propagate in the central nervous system (13, 21, 25). *vpr* may perform a function needed in these tissues. Alternatively, *vpr* may exert a function not readily measured in the tissue culture system. Nonpathogenic simian immunodeficiency virus isolated from African green monkey lacks the *vpr* open reading frame (9).

At present, the exact nature of *vpr* is not clear. *vpr* can affect any step of the virus growth cycle (adsorption, penetration, uncoating, transcription, mRNA processing, translation, protein processing, assembly, or maturation) and

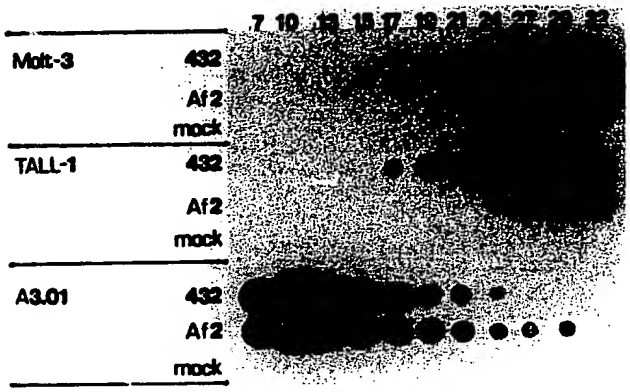


FIG. 4. Growth kinetics of a *vpr* mutant (Af2) and wild-type virus (432) in three human lymphocyte cell lines, Molt-3, TALL-1, and A3.01, by RT assays. Values at the top indicate day after infection. Equivalent dose ($5 \times 10^4$ cpm) was used for infection. Molt-3 and TALL-1 lines were obtained from the Japanese Cancer Research Resources Bank.

mutants exhibiting the phenotype described here. Several characteristics of *vpr* mutants should be pointed out. No significant difference in the transient expression of either wild-type or *vpr* mutant plasmids was observed by immunoblotting or electron microscopy (N. Ono, K. Ogawa, A. Adachi, and S. Ueda, manuscript in preparation), and RT activities in the culture fluids were indistinguishable. Growth kinetics of the *vpr* mutant is clearly distinct from those of *vif* and *vpu* mutants. These results suggest that HIV *vpr* is not important in the transmissibility, in the regulation of viral gene activity, or in the assembly and release of progeny virions. However, detailed genetic analysis coupled with biochemical study needs to be carried out to determine the function of *vpr*. In this respect, we have generated mutants affecting other genes, and complementation experiments are in progress in our laboratory. A comparative functional study on *vpr* of HIV-2 (12) is also important to evaluate this question further.

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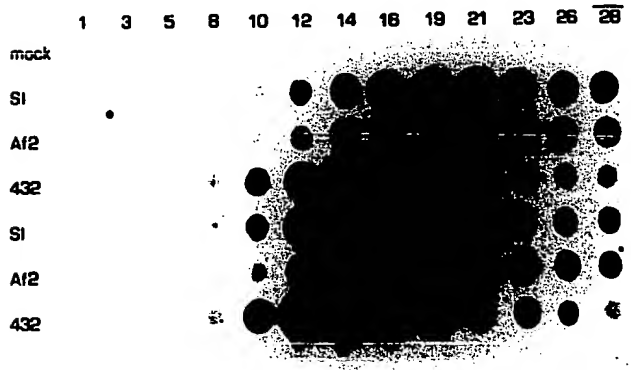


FIG. 5. Effect of input multiplicities on growth kinetics of *vpr* mutants (Af2 and Sl) and wild-type virus (432) in A3.01 cells by RT assays. Values at the top indicate day after infection. Input amounts of virus used for infection were $3 \times 10^3$ cpm (from 2nd line 2 to line 4) and $3 \times 10^4$ cpm (from line 5 to line 7).

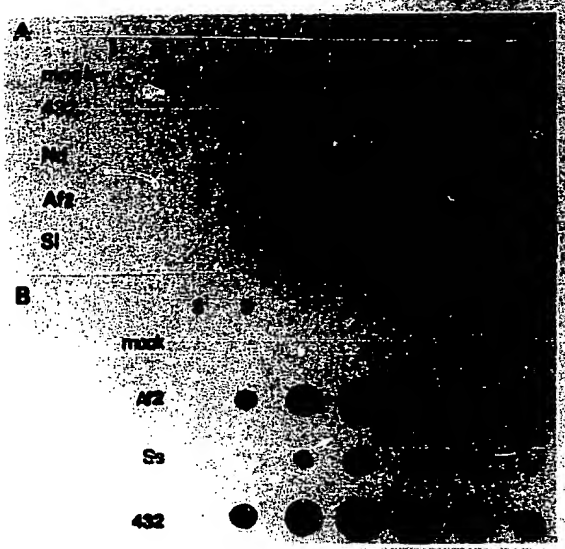


FIG. 6. Growth kinetics of *vif* (Nd), *vpr* (Af2), and *vpu* (Ss) mutants and wild-type virus (432) in A3.01 cells by RT assays. Values at the top indicate day after infection. Input dose for infection was $3 \times 10^4$ cpm. Experiments in parts A and B were carried out independently.

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# Viral Protein R of Human Immunodeficiency Virus Types 1 and 2 Is Dispensable for Replication and Cytopathogenicity in Lymphoid Cells

DOUGLAS DEDERA, WEN HU, NANCY VANDER HEYDEN, AND LEE RATNER*

*Departments of Medicine and Microbiology & Immunology, Washington University, St. Louis, Missouri 63110*

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**Viral protein R (VPR) is conserved in human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2). To assess its function, we have constructed mutations within the *vpr* coding regions of HIV-1 and HIV-2 predicted to express truncated VPR products. Infectious virus was produced by each proviral clone and showed similar replication kinetics and cytopathogenicity when compared with the corresponding parental proviral clone.**

The human immunodeficiency virus type 1 (HIV-1) genome includes three genes, *gag*, *pol*, and *env*, encoding virion proteins, and six genes encoding proteins which potentially regulate virus replication (6, 13). The regulatory proteins include transactivator (TAT), a positive-feedback regulator; negative factor (NEF), a negative-feedback regulator; regulator of virus production (REV), a differential regulator of virion and regulatory proteins; virion infectivity factor (VIF), which is needed for infection of certain cell lines (3); viral protein U (VPU), whose function is unknown; and viral protein R (VPR), which was analyzed in this study. HIV-2 encodes an additional protein not present in HIV-1, viral protein X (VPX), which is dispensable for replication and cytopathic effects (5, 7, 8, 17; our unpublished data).

The *vpr* gene is found in the central portion of lentivirus genomes. In HIV-1 it overlaps *vif* at its 5′ end, and in some HIV-1 strains it overlaps *tat* at its 3′ end (16). In different HIV-1 strains, the predicted protein products are 78 to 96 amino acids long and vary by 0 to 7% of amino acids within the first 78 amino acids. Thirty-four percent of HIV-1-infected individuals and no uninfected individuals possess antibodies reactive with a recombinant VPR product. This provides evidence for expression of the gene product in vivo.

In HIV-2, *vpr* follows *vif* and *vpx* and precedes *tat*; it is predicted to encode a protein of 105 amino acids (5). The VPR products of HIV-1 and HIV-2 are well conserved, with 52% amino acid homology, only slightly less than that of GAG and POL products (16). The *vpr* gene is also found in simian immunodeficiency virus from rhesus macaques (1) but not from African green monkeys (4). A similar sequence is also found in the visna virus genome (15).

To analyze the function of the HIV-1 VPR product, we constructed three oligonucleotide-directed mutations (15) by using a functional proviral clone, pX (2, 12). Oligonucleotides were synthesized and used for mutagenesis (9) to create termination codons at positions 3, 23, and 32 of the *vpr* gene product. The mutation at codon 3 was created by a cytosine-to-thymidine transition at nucleotide position 5145 of the parental genome, resulting in a 2-amino-acid VPR product. At codon 23, a termination codon and a unique *Xba*I site were created by inserting a cytosine residue between positions 5204 and 5205, resulting in a predicted VPR product of 22 amino acids. At codon 32, a termination codon and a unique *Xba*I site were created by the insertion of a cytosine residue between positions 5230 and 5231, resulting in a predicted VPR product of 31 amino acids. Another mutation was constructed by digestion at the *Nco*I site at position 5256 and insertion of four nucleotides with *Escherichia coli* DNA polymerase I Klenow fragment, causing a frameshift mutation after VPR residue 40, which truncates the protein and adds three amino acids. These mutants are designated pR2, pR22, pR31, and pR40, respectively. Each mutation was confirmed by nucleotide sequencing (14).

A mutation in a functional HIV-2 proviral clone (pSE) (10; our unpublished results) was constructed by a guanosine-to-thymidine transversion at position 5700, thus introducing a termination codon at VPR residue 7 and eliminating a *Sac*I restriction enzyme site. This clone is designated pMR7. An additional clone, designated pMRR, predicted to encode a full-length VPR product, was constructed with a change of an adenosine to a cytosine at position 5738, introducing a *Sma*I restriction site.

Virus production from each HIV-1 proviral clone was assessed by transfection of COS-1 or Jurkat cells and measurement of soluble p24 antigen production by enzyme-linked immunosorbent assay (Du Pont Co.). No significant differences in virus production were detected in cells transfected with pX compared with those transfected with pR2, pR22, pR31, or pR40 (results not shown). Furthermore, no differences were noted in TAT production by these clones as measured by their ability to *trans*-activate HIV-1 long terminal repeat-directed gene expression (results not shown).

To produce virus stocks from each proviral clone, we cocultivated H9 or CEM lymphoid cells with transfected COS-1 cells. Total cellular DNA was isolated from the infected cells and analyzed by Southern blot hybridization (Fig. 1). These data demonstrate that there are equivalent levels of proviral DNA in each infected cell line, as shown by the similar intensity of the hybridization signals (Fig. 1a, lanes 1 to 4; Fig. 1b, lanes 1 and 2; Fig. 1c, lanes 1 and 2; Fig. 1d, lanes 4 and 5; Fig. 1e, lanes 2 and 3). The presence of the mutation in R22 (Fig. 1a) and R31 (results not shown) was confirmed by the demonstration of the presence of an *Xba*I site, as indicated by the presence of a 2.8-kilobase (kb) DNA fragment after digestion with *Bam*HI and *Xba*I (Fig. 1a, lane

* Corresponding author.

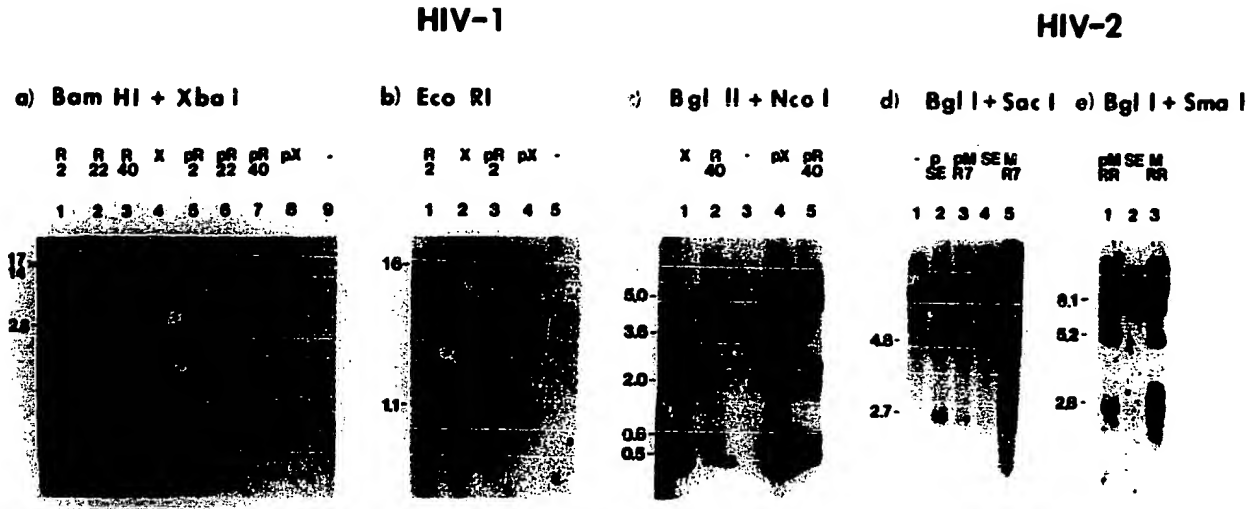


FIG. 1. Analysis of DNA sequences in HIV-1- and HIV-2-infected cells. Total cellular DNA was isolated from H9 cells infected with X, R2, R22, or R40 (a to c) or CEM cells infected with SE, MRR, or MR7 (d and e). Each DNA sample (10 μg) was digested with either *Xba*I and *Bam*HI (a), *Eco*RI (b), *Nco*I and *Bgl*II (c), *Bgl*I and *Sac*I (d), or *Bgl*I and *Sma*I (e). Controls include DNA from uninfected H9 or CEM cells or the same DNA to which 0.1 ng of cloned plasmid DNA from each proviral clone was added. Blots were hybridized with hexamer primer-labeled probes that include nucleotides 222 to 9213 of HIV-1 (a to c) or nucleotides 503 to 8598 of HIV-2 (d and e). Numbers to the left of each panel represent the size of the DNA fragments in kilobases.

2). The mutation in R40 was shown by the loss of the *Nco*I site (Fig. 1c, lane 2). The mutation in MRR was confirmed by the presence of a *Sma*I site demonstrated by the cleavage of the 8.1-kb DNA fragment to 5.2- and 2.8-kb fragments with *Bgl*I and *Sma*I (Fig. 1e, lane 3). The mutation in MR7 was confirmed by demonstrating the loss of a *Sac*I site in the polymerase chain amplified product of DNA from the infected cells (results not shown). These data suggest that fewer than 10% of the viral genomes have reverted.

Results of Southern blot analysis of restriction enzyme digests of the recombinant proviral DNA clones are also presented for comparison (Fig. 1a, lanes 5 to 8; Fig. 1b, lanes 3 and 4; Fig. 1c, lanes 4 and 5; Fig. 1d, lanes 2 and 3; Fig. 1e, lane 1). It should be noted that *Eco*RI digestion of plasmids pR2 and pX generates hybridizing bands of 16.0 and 1.1 kb, whereas the *Eco*RI digestion products of infected-cell DNA are about 9.0 and 1.1 kb, owing to the loss of plasmid DNA sequences (Fig. 1b).

Titers of virus stocks from conditioned medium of the infected H9 cells were determined by reverse transcriptase (11) and soluble p24 antigen assays. Equivalent amounts of undiluted, 5-fold-diluted, or 25-fold-diluted virus were then used to infect Molt-3 cells (Fig. 2a). Similar infectivity and replication rates were seen in viruses with or without an intact *vpr* gene, as demonstrated by a similar production of reverse transcriptase over time at each dilution. Furthermore, no alterations in cytopathogenicity were detected, as measured by cell killing (Fig. 2b) or syncytium production

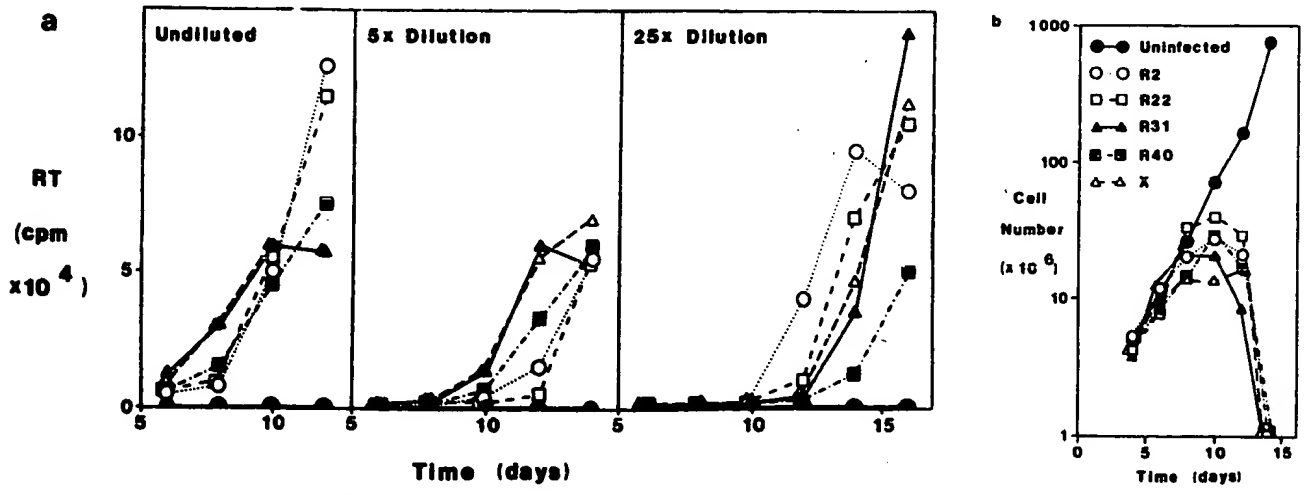


FIG. 2. Viruses from HIV-1 VPR mutant clones demonstrate similar infectivity, replication, and cytopathogenicity to virus from the parental clone. Equivalent amounts of X, R2, R22, R31, and R40, as determined by reverse transcriptase and soluble p24 antigen measurements, were used to infect Molt-3 cells by using an undiluted, 5-fold-diluted, or 25-fold-diluted virus stock. (a) Reverse transcriptase activity was measured with eightfold-concentrated samples of conditioned media at the indicated time points. (b) The number of cells in each culture infected with undiluted virus stocks was determined with a hemacytometer.

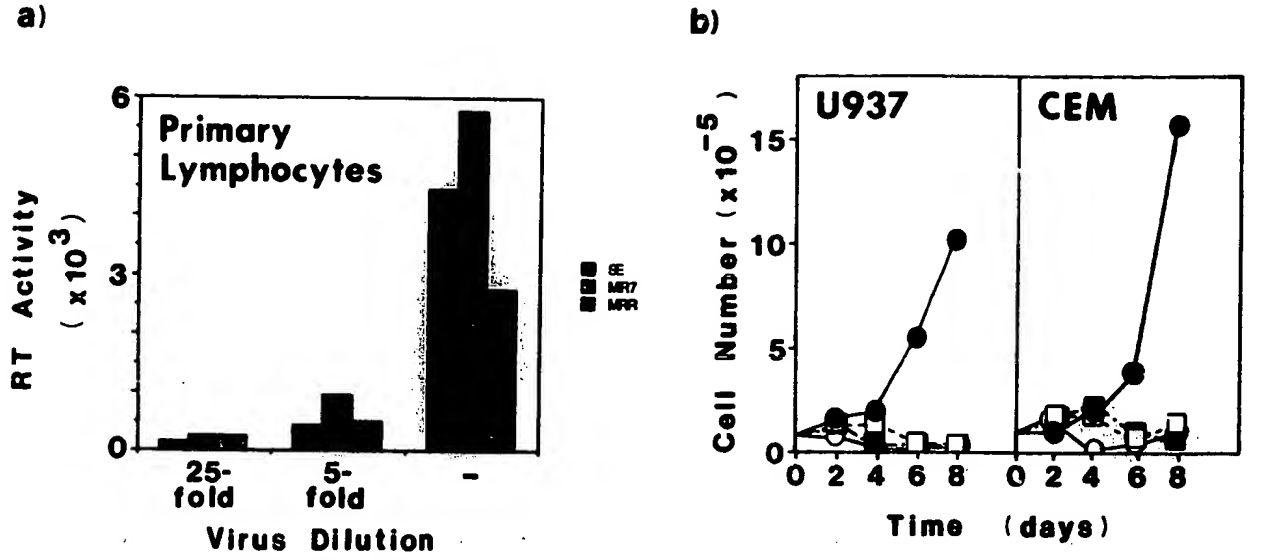


FIG. 3. Viruses from HIV-2 VPR mutant and parental clones demonstrate similar infectivity, replication, and cytopathogenicity. (a) Equivalent amounts of SE, MR7, and MRR virus, as determined by reverse transcriptase measurements, were used to infect primary human lymphocytes using an undiluted, 5-fold-diluted, or 25-fold-diluted virus stock. Reverse transcriptase activity was measured 2 days later. These values were more than 10-fold higher than those of the initial virus inocula. (b) Equivalent amounts of virus from COS-1 cells transfected with pSE (□), pMR7 (○), or pMRR (■) and cocultivated with CEM cells were used to infect U937 or CEM cells. Similar amounts of conditioned media from CEM cells cocultivated with COS-1 cells transfected with a pUC plasmid (pUC9) which lacks HIV sequences were used as a negative control (●). The cell number in 0.4-ml cultures in a 96-well plate were determined at each of the indicated time points with a hemacytometer.

(results not shown). Similar infectivity, replication, and cytopathic effects were found with R2, R22, R31, R40, and X in H9, Molt3 and CEM cells and with R40 and X in U937, SUP T1, and peripheral blood mononuclear cells (results not shown).

The HIV-2 VPR mutant, MR7, also demonstrated similar virus production to that of SE and MRR after transfection of COS-1 cells (results not shown). Virus obtained from MR7 infected and replicated at a similar level to SE and MRR in CEM, Sup T1, U937, and H9 cell lines (results not shown) and peripheral blood mononuclear cells (Fig. 3a). No differences in syncytium production (data not shown) or cell killing (Fig. 3b) were noted for MR7-, SE-, and MRR-infected cells.

These data demonstrate that *vpr* is dispensable for HIV-1 and HIV-2 infectivity, replication, and cell killing. Although the VPR protein is synthesized in infected individuals (16), currently available antisera have not detected VPR in infected cells. It is possible that VPR has subtle regulatory effects on the virus life cycle, which may not have been detected in the current study. Furthermore, although a broad range of cell lines and normal peripheral blood lymphocytes were examined in this study, effects of VPR in other cell types cannot be excluded. Another possibility is that the *vpr* genes from the particular HIV-1 or HIV-2 clones used in this study were partially or completely inactive. Alternatively, VPR may regulate virus replication in vivo in ways that are not detectable in our tissue culture models.

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# Human Immunodeficiency Virus *vpr* Product Is a Virion-Associated Regulatory Protein

ERIC A. COHEN,* GHASSAN DEHNI, JOSEPH G. SODROSKI, AND WILLIAM A. HASELTINE

*Division of Human Retrovirology, Dana-Farber Cancer Institute, Department of Pathology, Harvard Medical School, 44 Binney Street, Boston, Massachusetts 02115*

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**The *vpr* product of the human immunodeficiency virus type 1 (HIV-1) acts in *trans* to accelerate virus replication and cytopathic effect in T cells. Here it is shown that the HIV-1 viral particle contains multiple copies of the *vpr* protein. The *vpr* product is the first regulatory protein of HIV-1 to be found in the virus particle. This observation raises the possibility that *vpr* acts to facilitate the early steps of infection before de novo viral protein synthesis occurs.**

The *gag*, *pol*, and *env* genes of human immunodeficiency virus type 1 (HIV-1) encode the structural and replicative proteins that are incorporated into the virus particle. HIV-1 specifies at least six additional proteins which regulate viral replication (Fig. 1). Two of these genes, *tat* and *rev*, are essential for virus replication. The remaining genes, *nef*, *vif*, *vpu*, and *vpr*, are not required for virus replication, although mutations in these genes alter the replication properties of the virus (3). *vpr* was recently demonstrated to accelerate the replication and the cytopathic effect of HIV-1 in $CD4^+$ T cells (2, 5). *vpr* was also shown to specify a 15-kilodalton (kDa) protein that acts in *trans* to increase expression of viral proteins. *vpr* also stimulates expression of heterologous genes driven by the HIV-1 long terminal repeat (LTR) as well as other promoters (2).

To determine whether the *vpr* protein is incorporated into the virus particle, $CD4^+$ T cells were transfected with infectious proviruses isogenic except for the expression of *vpr*. Cells were transfected by the DEAE-dextran technique (8). The provirus that expresses the full-length *vpr* protein of 96 amino acids is designated $HXBRU^+$. A provirus isogenic except for a frameshift mutation in *vpr* that is predicted to terminate the product at amino acid 40 was used as a control ($HXBRU^-$) (2).

Eight days posttransfection, both cultures were metabolically labeled with 100 μCi of [$^{35}$S]cysteine per ml for 8 h as described elsewhere (9). Cells were collected by centrifugation, washed once with phosphate-buffered saline, and lysed with 750 μl of a buffer which contained 0.05 M Tris hydrochloride (pH 7.0), 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate. The medium containing the virus particle was centrifuged at 800 × *g* and passed through a 0.22-μm-pore-size filter to prevent cell contamination of the supernatant. Cell-free supernatants were then centrifuged for 1 h at 16,000 rpm in an Eppendorf microcentrifuge.

Lysates were prepared from both the labeled transfected cells and from the virus pellets. The cell and viral lysates were immunoprecipitated as described previously (9) with antiserum from a patient with acquired immune deficiency syndrome (AIDS) which recognized a broad spectrum of viral structural proteins. The lysates were also precipitated with a monospecific anti-*vpr* serum. The rabbit *vpr* antiserum used was raised against a synthetic peptide corresponding to the amino-terminal 19 amino acids of the *vpr* protein. As described previously (2), this antiserum recognizes a 15-kDa protein in cells producing the *vpr* protein (Fig. 2A, lane 1). The peptide used to raise the antiserum competed for recognition of the 15-kDa protein (lane 2). The *vpr* product was not detected in cells producing the $HXBRU^-$ virus (lane 4).

The *vpr* peptide antiserum also recognized the 15-kDa protein in the lysates of pelleted virus prepared from cells producing the $HXBRU^+$ virus (Fig. 2B, lane 1). No *vpr* product was detected in viruses pelleted from cultures producing the $HXBRU^-$ virus (Fig. 2B, lane 3). In addition, a protein of approximately 9 kDa of molecular mass was detected only in viruses pelleted from cultures producing the $HXBRU^+$ and $HXBRU^-$ viruses with both the *vpr* peptide antiserum and the AIDS patient serum (Fig. 2B, lanes 1 to 4). The recognition of this 9-kDa protein was not competed for by the *vpr* peptide, suggesting a nonspecific reaction (data not shown). These results indicate that the *vpr* protein is associated with the virus particle. The association appears to

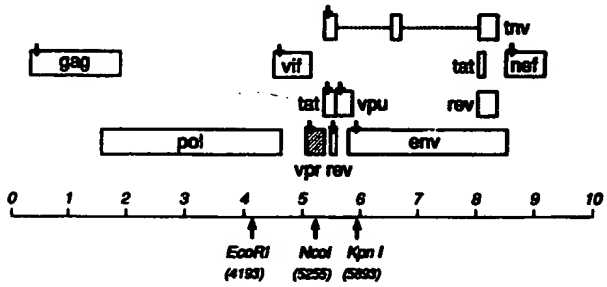


FIG. 1. Genetic organization of HIV-1. Arrows indicate the initiator AUG codons in viral genes. To generate a $vpr^+$ provirus, a segment of the HXBc2 provirus (6) located between the C terminus of *pol* (*Eco*RI at nucleotide position 4193; +1 is the site of initiation of transcription) and the N terminus of *env* (*Kpn*I site at nucleotide 5893), a region that contains the entire *vpr* coding sequence (▨), was replaced with the corresponding segment derived from the closely related BRU provirus (10). The genotype of the resultant provirus ($HXBRU^+$) is 5′ $LTR_{HXBc2}$ $gag^+_{HXBc2}$ $pol^+_{HXBc2}$ $vif^+_{BRU}$ $vpr^+_{BRU}$ $tat^+_{BRU}$ $rev^+_{BRU}$ $vpu^+_{BRU}$ $env^+_{HXBc2}$ $nef_{HXBc2}$ 3′ $LTR_{HXBc2}$. Introduction of a frameshift at an *Nco*I site (nucleotide position 5255) in $HXBRU^+$ generated a provirus expressing a truncated *vpr* product ($HXBRU^-$) (2). The location of the recently identified coding sequences of the *tat-env-rev* (*tnv*) fusion protein is shown (7a).

* Corresponding author.

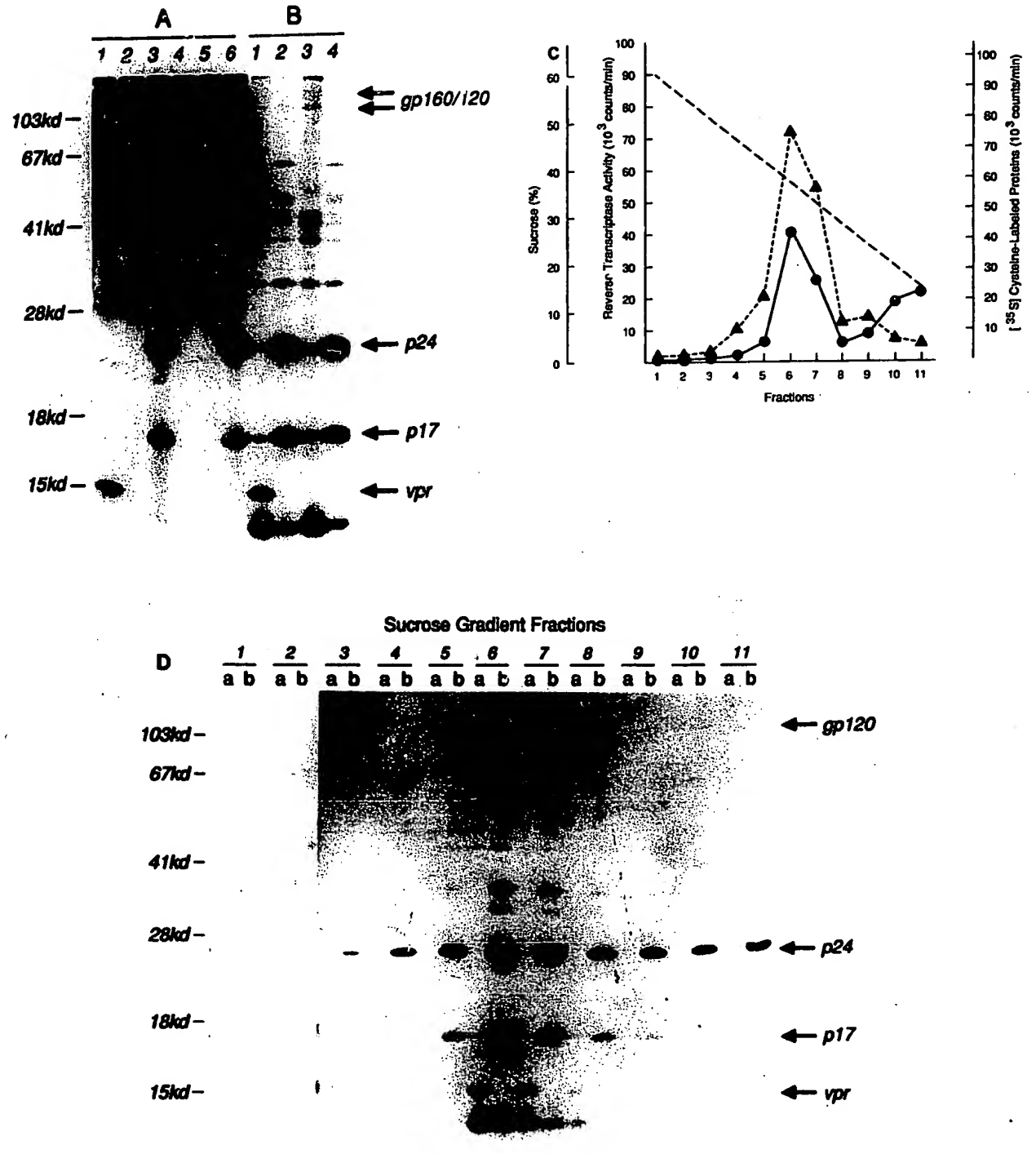


FIG. 2. Detection of the *vpr* product in the extracellular virions. (A) Lysates prepared from [$^{35}$S]cysteine-labeled Jurkat cells transfected with either HXBRU$^+$ (lanes 1 to 3) or HXBRU$^-$ (lanes 4 to 6) provirus were immunoprecipitated with *vpr* peptide antiserum (lanes 1 and 4), *vpr* peptide antiserum preincubated with 500 μmol of peptide (lanes 2 and 5), or AIDS patient serum (lanes 3 and 6). (B) Lysates of pelleted virus prepared from Jurkat cells transfected with either HXBRU$^+$ (lanes 1 and 2) or HXBRU$^-$ (lanes 3 and 4) provirus were immunoprecipitated with *vpr* peptide antiserum (lanes 1 and 3) or AIDS patient serum (lanes 2 and 4). (C) Pelleted virions prepared from [$^{35}$S] cysteine-labeled HXBRU$^+$-transfected Jurkat cells were suspended in 400 μl of TNE, layered on a 15 to 60% (wt/vol) sucrose gradient, and centrifuged at 45,000 rpm for 3 h as described previously (1). Eleven fractions (400 μl each) were collected. Reverse transcriptase assays (7) were performed on 50-μl portions of each fraction (▲). Ten microliters of each fraction was counted in a liquid scintillation counter to determine the amount of [$^{35}$S]cysteine-labeled proteins (●). (D) Immunoprecipitates of either 240 or 100 μl of each sucrose gradient fraction with *vpr* peptide antiserum (a) or AIDS patient antiserum (b), respectively. kd, Kilodaltons.

be specific, since in similar experiments using this and other proviruses, monospecific antiserum to the *tat*, *rev*, *vpu*, and *nef* proteins did not detect these proteins in the pelleted fraction of the viruses (data not shown).

To rule out cell contamination in the supernatant, the suspended HXBRU$^+$ virus pellet was layered onto a sucrose gradient as described previously (1). The virus was suspended in 400 μl of a buffer (TNE) which contained Tris (10 mM; pH 7.4), 100 mM NaCl, and 1 mM EDTA. A 4-ml linear sucrose gradient (15 to 60%, wt/vol) spun at 45,000 rpm for 3 h was used. Eleven fractions of 400 μl each were collected. The amount of [$^{35}$S]cysteine-labeled protein in each fraction and the reverse transcriptase activity were determined (Fig. 2C) (7). Additionally, each fraction was precipitated with either an AIDS patient antiserum or the monospecific *vpr* antiserum (Fig. 2D). The *vpr* protein cosedimented with virus particles by all measures used (Fig. 2C and D). The peak of *vpr* protein corresponds to the peak of reverse transcriptase activity, the peak of [$^{35}$S]cysteine-labeled proteins in the gradient, and the peak of virion proteins as detected by AIDS patient antiserum.

The amount of radioactivity in the bands corresponding to p24, p17, and *vpr* p15 protein in the immunoprecipitates of cell lysates and virus particles was determined. The results showed that the ratios of the proteins associated with the viral particles and the cells were roughly equivalent, between 5:1 and 10:1 (data not shown). Although it is difficult to calculate stoichiometry from such measurements because the efficiency of immunoprecipitation may vary for each protein, the results indicate that multiple *vpr* proteins were incorporated into each virion.

The results of these experiments demonstrate that the *vpr* protein is incorporated into cell-free virus particles. The *vpr* protein is the first regulatory product of HIV-1 to be found associated with viral particles. Experiments similar to these failed to detect the *tat*, *rev*, *vpu*, or *nef* protein associated with virus particles (E. A. Cohen, J. G. Sodroski, and W. A. Haseltine, unpublished observations). The *vpx* protein of HIV type 2 and of the simian immunodeficiency virus was also recently reported to be associated with the virus particles (12). There is no similarity in amino acid sequence between the *vpr* and *vpx* proteins.

The mechanism by which *vpr* becomes associated with the virus particle is unknown. Other capsid proteins are made as *gag* or *gag-pol* precursors and assembled as units into the budding particle (4, 11). The *vpr* protein is not known to be part of such a larger precursor and must have an independent means of association with the nascent particle.

The presence of a transactivating protein in the mature virus particles suggests that this protein plays a role in early steps of virus infection. Possible roles for such a protein include facilitation of the reverse transcription reaction, stabilization of RNA, stabilization of RNA-DNA or DNA-DNA structures, facilitated migration of the proviral DNA to the nucleus, and facilitated integration. The ability of the *vpr* protein to increase the rate of expression of the HIV-1 and other promoters raises the possibility that the virion-associated *vpr* may activate early transcription of the HIV-1 provirus. The *vpr* product may also alter cellular expression before viral RNA transcription begins. Alteration of the cell environment by *vpr* carried into the cell by the infecting virus may alter the initial rate of HIV-1 RNA accumulation before viral proteins are made de novo.

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# Human Immunodeficiency Virus Type 1 Viral Protein R Localization in Infected Cells and Virions

YUH-LING LU, PAUL SPEARMAN, AND LEE RATNER*

*Departments of Medicine and Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110*

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**The subcellular localization of human immunodeficiency virus type 1 (HIV-1) viral protein R (Vpr) was examined by subcellular fractionation. In HIV-1-infected peripheral blood mononuclear cells, Vpr was found in the nuclear and membrane fractions as well as the conditioned medium. Expression of Vpr without other HIV-1 proteins, in two different eukaryotic expression systems, demonstrated a predominant localization of Vpr in the nuclear matrix and chromatin extract fractions. Deletion of the carboxyl-terminal 19-amino-acid arginine-rich sequence impaired Vpr nuclear localization. Indirect immunofluorescence confirmed the nuclear localization of Vpr and also indicated a perinuclear location. Expression of Vpr alone did not result in export of the protein from the cell, but when coexpressed with the Gag protein, Vpr was exported and found in virus-like particles. A truncated Gag protein, missing the p6 sequence and a portion of the p9 sequence, was incapable of exporting Vpr from the cell. Regulation of Vpr localization may be important in the influence of this protein on virus replication.**

The human immunodeficiency virus type 1 (HIV-1) genome is more complex than those of murine and avian retroviruses. In addition to the basic functions encoded by *gag*, *pol*, and *env*, the HIV-1 genome includes at least six additional genes with distinct regulatory roles (see references 31 and 39 for reviews). Two of these regulatory genes, *tat* and *rev*, are essential for virus gene expression. The remaining genes, *vpr*, *vpu*, *vif*, and *nef* are dispensable for virus replication in tissue culture, but mutations of these genes alter the replication properties of the virus.

HIV-1 *vpr* encodes a protein (viral protein R [Vpr]) of 96 amino acids (27). Previous studies have shown that the *vpr* products can increase the rate of replication of the virus and accelerate its cytopathic effects in T-cell lines and in peripheral blood mononuclear cells (PBMCs) (6, 8, 28, 29). Cohen and colleagues suggested that *vpr* increased gene expression from the HIV-1 promoter, as well as a wide range of other promoters, but the mechanism of this effect remains to be determined (7). *vpr* is also found in the genomes of HIV-2 and several strains of simian immunodeficiency virus (SIV) (5, 14). The activity of the HIV-2 and SIV *vpr* gene products appears to be similar to that of HIV-1 *vpr* (16, 35). Furthermore, $SIV_{mac}$ *vpr* is important for the development of an AIDS-like disease in rhesus macaques (22).

HIV-1, HIV-2, and SIV *vpr* gene products have 26 to 36% amino acid identity (40). Certain features of the Vpr proteins among different HIV isolates are highly conserved, including the presence of a single cysteine residue at amino acid position 76 of HIV-1 Vpr, a predicted amphipathic alpha-helical loop in the N-terminal portion of the protein, and the presence of an arginine-rich carboxyl-terminal tail.

Vpr is packaged within the HIV-1 virion (6, 45). Similar findings have been reported for $SIV_{mac}$ Vpr (44). The Vpr protein is the only regulatory product of HIV-1 found in virus particles, though the homologous *vpx* gene products of HIV-2 and $SIV_{mac}$ are also associated with virus particles (17–19). However, the subcellular distribution of Vpr and the mechanism of incorporation into virus particles are unclear. In this study, the subcellular localization of Vpr in HIV-1-infected PBMCs and in two different *vpr* expression systems in mammalian cells was examined by subcellular fractionation and indirect immunofluorescence techniques. The role in cellular localization of the carboxyl-terminal arginine-rich sequence of Vpr was specifically studied. Lastly, the effects of Gag coexpression on Vpr export and incorporation into virus particles were examined.

## MATERIALS AND METHODS

**Cell lines and culture.** COS-7 cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 1 mM pyruvate, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. BSC40 cells were maintained in the same medium. PBMCs were purified from normal human leukocytes by centrifugation onto Ficoll. After 3 days of stimulation with phytohemagglutinin (15 μg/ml; Sigma), PBMCs were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 4 mM glutamine, 50 U of recombinant interleukin 2 (Cetus) per ml, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

**Vpr and Gag expression plasmids.** HIV-1 nucleotides 5558 to 5869 (numbered according to reference 27) encompassing the pNL4-3 *vpr* gene was obtained by the polymerase chain amplification reaction, using primers AATACCATGGAA CAAGCCCCAGAAGA and GATGCTTCCAGGGATCCGT CTAGGATCTACTG. The reaction product was digested with *Nco*I and *Bam*HI and cloned into pTM3 (designated here pTM) (12, 26), to produce pTM-VPR. The *Nco*I-*Bam*HI fragment of pTM-VPR was cloned between the *Sal*I and *Sac*I sites of pSRalpha (25) after blunt ending with T4 DNA polymerase, in the correct orientation (pSR-VPRs) and in the incorrect or antisense orientation (pSR-VPRa) (see Fig. 2A). The CRST mutant clone was constructed by digestion of pNL4-3 with SalI, at nucleotide 5786, and blunt ending with the Klenow fragment of DNA polymerase I. It was then cloned

* Corresponding author.

into the pTM vector by the strategy used to construct pTM-VPR.

An *Nco*I-*Nco*I fragment from plasmid pGG1 (3, 32), containing nucleotides 789 to 5674, was cloned into the *Nco*I site of plasmid pTM3, to produce plasmid pTM-GAG-POL. This clone contains the *gag* and *pol* open reading frames. Expression of the *pol* gene was abrogated by frameshift mutation at the *Bcl*I site at nucleotide 2428 in the 5′ portion of *pol* to produce pTM-GAG. Plasmid pTM-GAG(p41) was constructed from pTM-GAG-POL by frameshift mutation at the *Apa*I site at nucleotide 2005, using T4 DNA polymerase I. This results in a termination codon at nucleotide 2058 after the first Cys-His box coding region of p9.

**Vpr and Gag p24 antisera.** A New Zealand White rabbit was inoculated with complete Freund's adjuvant containing 200 μg of Vpr protein, synthesized according to the sequence of HIV-1 strain LAI and kindly provided by H. Gras-Masse (13). Booster doses of 200 μg of Vpr in incomplete Freund's adjuvant were given at 3, 6, 9, and 18 weeks after the initial inoculation. A New Zealand White rabbit was inoculated with complete Freund's adjuvant containing 100 μg of recombinant p24 protein (provided by American Biotechnology through the NIH AIDS Research and Reference Reagent Program), and 100-μg booster doses were given 2 and 4 weeks later.

**Virus infection.** HIV-1 virus stocks were generated by transfection of 60% confluent 10-cm-diameter COS-7 plates with 10 μg of recombinant proviral clone NLHXADA(GG) (41) and 2 μg of pCV1 (*tat* expression vector [1]) by the calcium phosphate precipitation method, followed 5 h later by 10% dimethyl sulfoxide shock for 2 min. The cells were washed twice with phosphate-buffered saline (PBS) before refeeding with 10 ml of fresh medium. Culture supernatants were harvested after 48 h and filtered (0.2-μm-pore-size Millipore filter). Five milliliters of culture supernatant was used to infect $5 \times 10^7$ PBMCs. Virus replication was monitored by determination of reverse transcriptase activity (30). PBMCs ($10^7$) were labeled for 20 h in 2 ml of leucine-free RPMI 1640 medium containing 200 μCi of [4,5-$^3$H]leucine and fractionated as described below. Mock-infected cultures were exposed to 5 ml of filtered culture supernatants from untransfected COS-7 cells.

**Transfection and radiolabeling of COS-7 cells.** COS-7 cells were grown to 60% confluence on 10-cm-diameter culture dishes and transfected with 15 μg of pSR-VPRs or pSR-VPRa by lipofection as recommended by GIBCO. Briefly, 15 μl of Lipofectin (GIBCO) was mixed with 3 ml of Opti-MEM I reduced-serum medium (GIBCO), and then 15 μg of DNA was added. The mixture was allowed to incubate at room temperature for 10 min before addition of the cells. Forty-eight hours after transfection, the cells were labeled with 4 ml of leucine-free DMEM containing 100 μCi of [4,5-$^3$H]leucine per ml for 40 h.

**Infection-transfection protocol for the vaccinia virus expression system.** BSC40 cells were grown to 90% confluence on 10-cm-diameter plates, infected for 1 h at 37°C with vTF7-3 (12, 26) at a multiplicity of infection of 10, and transfected with pTM vectors by the lipofectin transfection method. Four hours after transfection, the cells were labeled for 20 h with 3 ml of leucine-free DMEM containing 100 μCi of [4,5-$^3$H]leucine per ml.

**Subcellular fractionation.** Labeled cells were fractionated into membrane, cytosolic, postnuclear, and nuclear fractions as previously described (23), with minor modifications. Nuclei were further fractionated into nucleoplasm, chromatin extract, and nuclear matrix as described by Staufenbiel and Deppert (37). Cells were washed with ice-cold PBS and scraped in PBS. The cell pellet volume was measured and resuspended in 10 volumes of Dounce buffer (10 mM Tris-HCl [pH 7.5], 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM dithiothreitol). The cells were allowed to swell on ice for 10 min before disruption with 20 to 25 strokes of a Dounce homogenizer. A small aliquot was saved and mixed with an equal volume of 0.4% (wt/vol) trypan blue in PBS to examine cell disruption under phase microscopy. Dounce homogenization was continued until >99% cells were disrupted. The homogenate was centrifuged at 1,500 rpm for 10 min in a Beckman GS-6 rotor to generate the supernatant containing both the membrane and cytosolic fractions and the nuclear pellet.

The nuclear pellet was subsequently extracted by four steps. First, the nuclear pellet was resuspended in buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES-KOH; pH 7.4], 0.25 M sucrose, 0.2 mM PMSF, 0.5 mM dithiothreitol) supplemented with 0.1% (vol/vol) Triton X-100 and then incubated for 15 min on ice. The nuclei were pelleted at 1,500 rpm for 10 min, and the supernatant was designated the postnuclear wash fraction. Second, the pellet was resuspended in buffer A supplemented with 0.5% Nonidet P-40 and incubated for 30 min on ice. The nuclei were pelleted again at 1,500 rpm for 10 min, and the supernatant was designated the nucleoplasmic fraction. The latter procedure was repeated twice, and the supernatants were pooled. Third, the Nonidet P-40-extracted nuclear pellet was subjected to DNase I digestion (1% [vol/vol] Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM PMSF, and 50 μg of DNase I [Sigma] per ml in PBS) for 15 min at 37°C. Then an equal volume of 4 M NaCl was added, and incubation was continued for 30 min at 4°C. The sample was then subjected to centrifugation at 2,500 rpm for 10 min. The supernatant was designated the chromatin extract, and the pellet was resuspended in radioimmunoprecipitation assay (RIPA) buffer (1% [vol/vol] Triton X-100, 0.5% [wt/vol] deoxycholate, 0.1% [wt/vol] sodium dodecyl sulfate [SDS], and 0.2 mM PMSF in PBS) for 30 min on ice. The insoluble portion was removed by centrifugation at 1,000 rpm for 15 min. The supernatant was designated the nuclear matrix. The purity of the nuclei was examined by using a control cytosolic protein, β-galactosidase, expressed in the same cells by transfection of a cDNA expression clone. More than 98% of the β-galactosidase activity was found in the cytosol, as measured by enzymatic assay. Only 1.4% of the β-galactosidase activity was detected in the postnuclear wash fraction. No detectable activity was found in the purified nuclei.

For the membrane and cytosolic fractions, the salt concentration was adjusted to 0.15 M NaCl and then the preparations were fractionated by ultracentrifugation at 100,000 × *g* for 30 min. The supernatant was designated the cytosolic fraction. The pellet was washed with 1 M NaCl in PBS for 30 min on ice, and ultracentrifugation was repeated. The supernatant was designated the membrane wash fraction, and the membrane pellet was resuspended in RIPA buffer.

**Immunoprecipitation.** Equivalent proportions (volume/volume) of each of the subcellular fractions were precipitated overnight with 10% trichloroacetic acid at 4°C. The resulting pellets were washed in 70% ethanol, solubilized in sample buffer (0.125 M Tris-HCl [pH 6.8], 20% glycerol, 10% [vol/vol] 2-mercaptoethanol, 4% [wt/vol] SDS). An aliquot was taken for scintillation counting to determine relative labeled protein content.

Equivalent proportions (volume/volume) of each of the subcellular fractions were adjusted to 500 μl of RIPA buffer. Immunoprecipitation was performed by the addition to 500 μl of each fraction 5 μl of anti-Vpr antiserum or 5 μl of anti-Gag

antiserum. Incubation was continued overnight at 4°C. Twenty microliters of protein A-Sepharose beads (50% [vol/vol] in PBS) was added, and the mixture was incubated for 120 min at 4°C. Immunoprecipitates were collected at 500 × *g* for 3 min at room temperature and washed three times with RIPA buffer. The beads were resuspended in 30 μl of sample buffer. Samples were treated at 100°C for 10 min before SDS–12% polyacrylamide gel electrophoresis (PAGE), fixation for 30 min in 25% isopropanol–10% acetic acid, treatment with Amplify (Amersham), and autoradiography were performed. Band intensities were determined by densitometry.

**Immunofluorescence.** BSC40 cells ($10^4$) were plated on eight-well Lab-Tek chamber slides overnight. The cells were infected with vTF7-3 and transfected as described above. The cells were fixed with 2.5% (wt/vol) glutaraldehyde for 15 min and permeabilized with 0.2% (vol/vol) Triton X-100 for 6 min at room temperature. The cells were then blocked for nonspecific binding of immunoglobulin by incubation for 30 min with PBS containing 5% (wt/vol) nonfat dry milk and 0.1% (vol/vol) Tween 20. Slides were then incubated with rabbit anti-Vpr antibody (1:100 in Tween buffer [PBS with 0.5% Tween 20 and 1% bovine serum albumin]) and mouse monoclonal antihistone antibody (1:500 in Tween buffer; Chemicon) for 1 h at room temperature. The cells were washed several times with 0.3% (vol/vol) Triton X-100 in PBS and incubated at 4°C for 60 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G to detect Vpr and rhodamine-conjugated goat anti-mouse immunoglobulin G to detect histones. The slides were washed extensively with PBS and mounted in Aqua mount solution (Lerner Lab) containing 2.5% 1,4-diazobicyclo-[2,2,2]-octane (Sigma) to prevent photobleaching of the FITC signal. Slide preparations were examined on a Nikon fluorescence microscope equipped with appropriate filters and a microflex UFX camera system. Photographs were prepared by using Kodak T-MAX film, push processed to ASA 3200.

**Sucrose gradients.** BSC40 cells were infected, transfected, and labeled with [$^3$H]leucine as described above. Cellular debris was removed from the conditioned medium by centrifugation at 2,500 rpm for 15 min in a Beckman GS-6 rotor. Particles were concentrated by sedimentation through a 20% sucrose cushion prepared in PBS at 28,000 rpm for 90 min at 4°C in an SW28.1 rotor. Particles were resuspended in 200 μl of PBS, layered on a linear 20 to 60% sucrose gradient in PBS, and centrifuged in an SW28.1 rotor at 20,000 rpm for 16 h at 4°C. Fractions were collected from the top of the tube.

## RESULTS

**Localization of Vpr in HIV-1-infected PBMCs.** To examine the intracellular localization of Vpr, PBMCs, a natural target cell population, were chosen for HIV-1 infection. HIV-1 strain NLHXADA(GG) was chosen since it encodes a functional 96-amino-acid form of Vpr identical in amino acid sequence to that encoded by NL4-3 (27, 29, 42). Nine days after infection, the cells were labeled for 20 h with [$^3$H]leucine, lysed by Dounce homogenization, and then fractionated into nuclear, cytosolic, and membrane fractions by differential centrifugation. Each fraction was immunoprecipitated with a polyclonal rabbit anti-Vpr antiserum and subjected to SDS-PAGE (Fig. 1). A Vpr-specific protein of 14 kDa was detected in NLHX ADA(GG)-infected cells and conditioned medium but not in mock-infected cultures. This protein was not immunoprecipitated with a control antiserum obtained from the preblced serum of the same rabbit prior to inoculation with the synthetic

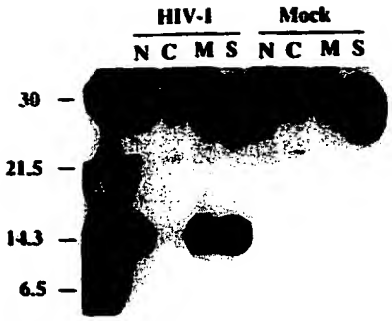


FIG. 1. Localization in PBMCs of Vpr expressed from infectious virus. PBMCs were infected for 9 days with HIV-1 strain NLHX ADA(GG) or were mock infected. The cells were labeled with [$^3$H]leucine, the medium was harvested (S), and the cells were fractionated into nuclear (N), cytosolic (C), and membrane (M) fractions as described in Materials and Methods. Equivalent portions of each fraction were immunoprecipitated with the anti-Vpr antiserum and analyzed by SDS-PAGE. Molecular mass markers are shown at the left in kilodaltons.

Vpr used for production of the anti-Vpr antiserum (not shown).

The majority of Vpr was found in the culture supernatant, consistent with previous reports that Vpr is virion associated (6, 45). Intracellular Vpr was found in both nuclear (26%) and membrane (20%) fractions. Vpr in the nuclear fraction was not due to contamination with unbroken cells, which made up <1% of the total cell population used in the fractionation experiments. Less than 4% of the Vpr was found in the cytosolic fraction. Approximately 50% of the labeled Vpr was found in the conditioned medium.

**Expression and localization of Vpr in mammalian cells.** To study the cellular localization of Vpr without the effect of other HIV-1 components, the NLHXADA(GG) *vpr* gene was cloned into a simian virus 40 (SV40)-based vector system, pSRalpha, in both the correct (pSR-VPRs) and incorrect or antisense (pSR-VPRa) orientations (Fig. 2A). The expression plasmid contains both the SV40 early enhancer (SV40-ori) and a human T-cell leukemia virus type I (HTLV-I) promoter with R and U5 elements of the HTLV-I long terminal repeat. This expression plasmid has previously been reported to achieve high levels of expression of a number of different lymphokine cDNAs in a variety of cell types (38) and to facilitate the expression of HIV-2 *vpx* in COS-1 cells (21).

pSR-VPRs and pSR-VPRa were transfected into COS-7 cells, and the cells were labeled with [$^3$H]leucine and separated into membrane, membrane wash, cytosolic, and nuclear fractions. Membranes loosely associated with nuclei were removed by a wash with 0.1% Triton X-100 and were designated the postnuclear wash. Soluble nucleoplasmic proteins were extracted with two successive washes in 0.5% Nonidet P-40, which permeabilizes the nuclear membrane (2). This method has previously been demonstrated to preserve overall nuclear and nucleolar architecture (33). The chromatin fraction was obtained by digestion of the resultant insoluble nuclear fraction with DNase I and by a subsequent wash in a high-salt buffer. This fraction contained all of the major histone proteins found in intact nuclei (not shown). The salt- and detergent-insoluble fraction was pelleted to yield the nuclear matrix fraction, which was solubilized in RIPA buffer.

The partition of Vpr during fractionation was examined by immunoprecipitation with the anti-Vpr antibody, SDS-PAGE, and densitometric quantitation (Fig. 2B). The chromatin frac-

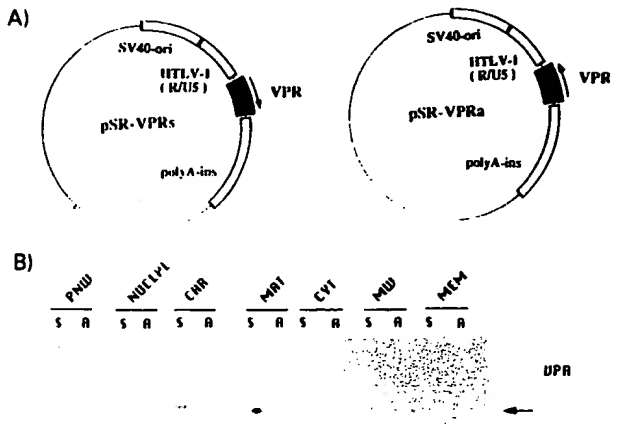


FIG. 2. Localization in COS-7 cells of Vpr expressed from pSR. (A) Vpr sense (pSR-VPRs) and antisense (pSR-VPRa) expression plasmids, which include a transcriptional enhancer (SV40-ori), a transcriptional promoter (HTLV-I long terminal repeat [R/U5]), and a polyadenylation insertion sequence (polyA-ins). (B) Subcellular fractionation of Vpr expressed in transfected and [$^3$H]leucine-labeled COS-7 cells from pSV-VPRs (S) and pSR-VPRa (A) in postnuclear wash (PNW), nucleoplasm (NUCLPL), chromatin (CHR), nuclear matrix (MAT), cytosol (CYT), membrane wash (MW), and membranes (MEM). Equivalent amounts of each fraction were immunoprecipitated with the anti-Vpr antiserum and analyzed by SDS-PAGE. The electrophoretic position of Vpr is shown by an arrow at the right.

tion included 17% of the intracellular labeled proteins and 44% of the total Vpr. The nuclear matrix included 1% of the intracellular labeled proteins and 56% of the total Vpr. Less than 1% of the total Vpr was found in the other cellular fractions.

**Truncation of the C terminus of Vpr impairs nuclear localization.** Most nuclear localization signals consist of a short stretch of positively charged amino acids (15). Interestingly, the C terminus of Vpr contains a high proportion of positively charged amino acids, including 7 arginine residues among the C-terminal 20 amino acids (Fig. 3A). To characterize the role of this C-terminal sequence, a vaccinia virus expression system was used to achieve high-level and rapid expression of Vpr. The NLHXADA(GG) *vpr* gene was cloned into pTM3, a plasmid utilizing a T7 promoter for heterologous gene expression. This plasmid was designated pTM-VPR. A carboxyl-terminal truncation mutant of pTM-VPR, pTM-CRST, was constructed by frameshift mutation at the *SalI* site. A recombinant vaccinia virus, vTF7-3, which encodes T7 RNA polymerase was used for expression in mammalian cells.

BSC40 cells were infected with vTF7-3 and transfected with pTM-VPR or pTM-CRST. Cells were labeled with [$^3$H]leucine, cell supernatants were harvested, and disrupted cells were fractionated into membrane, membrane wash, cytosolic, and postnuclear wash fractions and various nuclear fractions (nucleoplasmic proteins, chromatin, and nuclear matrix). Equivalent amounts of each fraction were immunoprecipitated with the anti-Vpr antiserum and analyzed by SDS-PAGE (Fig. 3B), and band intensities were quantitated by laser densitometry (Fig. 3C). The predominant Vpr product expressed from pTM-VPR had an electrophoretic mobility of a 14-kDa protein, with a minor band with a mobility of a 13-kDa protein. Vpr expressed from pTM-CRST electrophoresed as a 12-kDa protein, consistent with the removal of 17 amino acids from the carboxyl terminus.

Eighty-four percent of pTM-VPR-expressed Vpr was found in nuclear fractions, primarily the nuclear matrix and chromatin fractions (Fig. 3B [left] and C). This result is in agreement with the fractionation data with pSR-VPR-expressed Vpr (Fig. 2). Eight percent of pTM-VPR-expressed Vpr was tightly associated with the membrane fraction (Fig. 3B and C, MEM). The possible discrepancy in the amount of membrane association of Vpr expressed with the vaccinia virus expression system compared with the data obtained with pSR-VPR (Fig. 2) may be related to the significantly higher level of expression of Vpr with the vaccinia virus expression system than with the SV40 plasmid expression system. Only 4% of Vpr was found in the cytosol (Fig. 3B and C, CYT), and no detectable Vpr was released from cells into the cell supernatant.

Deletion of the arginine-rich C terminus of Vpr resulted in a dramatic shift of Vpr cellular localization (Fig. 3B [right] and C). Only 25% of the truncated Vpr was retained in the nuclear fraction. Furthermore, the distribution in nuclear fractions of pTM-CRST product was distinctly different from that of pTM-VPR, with the majority of the truncated protein in the nucleoplasm. Twenty-four percent of the pTM-CRST protein was in the postnuclear wash, compared with 3% of the pTM-VPR product. Thirty-eight percent of the mutant Vpr was found in the cytosol, compared with 4% of the parental Vpr. Similar amounts of pTM-CRST and pTM-VPR products were bound to membranes.

**Indirect immunofluorescence localization of Vpr.** Subcellular fractionation experiments indicated predominant localization of Vpr in the nucleus. To confirm these results, indirect immunofluorescence was performed with fixed cells. BSC40 cells were infected with vTF7-3 and then transfected with pTM-VPR or pTM. Vpr was detected by anti-Vpr rabbit antibody and visualized with FITC-conjugated anti-rabbit antibody. Intense immunofluorescence was observed in the majority of cells transfected with pTM-VPR (Fig. 4A, left), but no fluorescence was observed in cells transfected with the vector pTM alone (Fig. 4A, right) or if preimmune serum was used (not shown).

Four types of staining patterns were observed in four independent experiments in which 50 cells were randomly selected and enumerated. Sixty-two percent of the cells showed a diffuse nuclear and focal perinuclear staining pattern (Fig. 4B, middle). The nucleus is visualized by phase-contrast microscopy (Fig. 4B, left) and mouse antihistone and rhodamine-conjugated anti-mouse antibody (Fig. 4B, right). Twenty-seven percent of the cells showed focal perinuclear staining only. Six percent of the cells had diffuse perinuclear staining with intense immunofluorescence surrounding the nucleus. Four percent of the cells showed only diffuse nuclear staining.

**Influence of Gag protein on Vpr export and virion incorporation.** In HIV-1-infected cells, Vpr was found to be exported into the medium in virus particles (Fig. 1) (6, 45), though no export was found when Vpr was expressed in the absence of other virion components (Fig. 3). To assess the requirements for export, Vpr was coexpressed with the HIV-1 Gag p55 precursor protein by using plasmid pTM-GAG. BSC40 cells were infected with vTF7-3 and transfected with pTM-VPR alone, pTM-GAG alone, or both plasmids. No differences were noted in the electrophoretic mobility or quantity of the 14-kDa product in the cell lysates with pTM-VPR expressed in the presence or absence of pTM-GAG (Fig. 5A, left). The pTM-GAG product was primarily a 55-kDa protein, with smaller amounts of 43- and 41-kDa products. The latter proteins were found to be Gag proteins, since they did not react with a

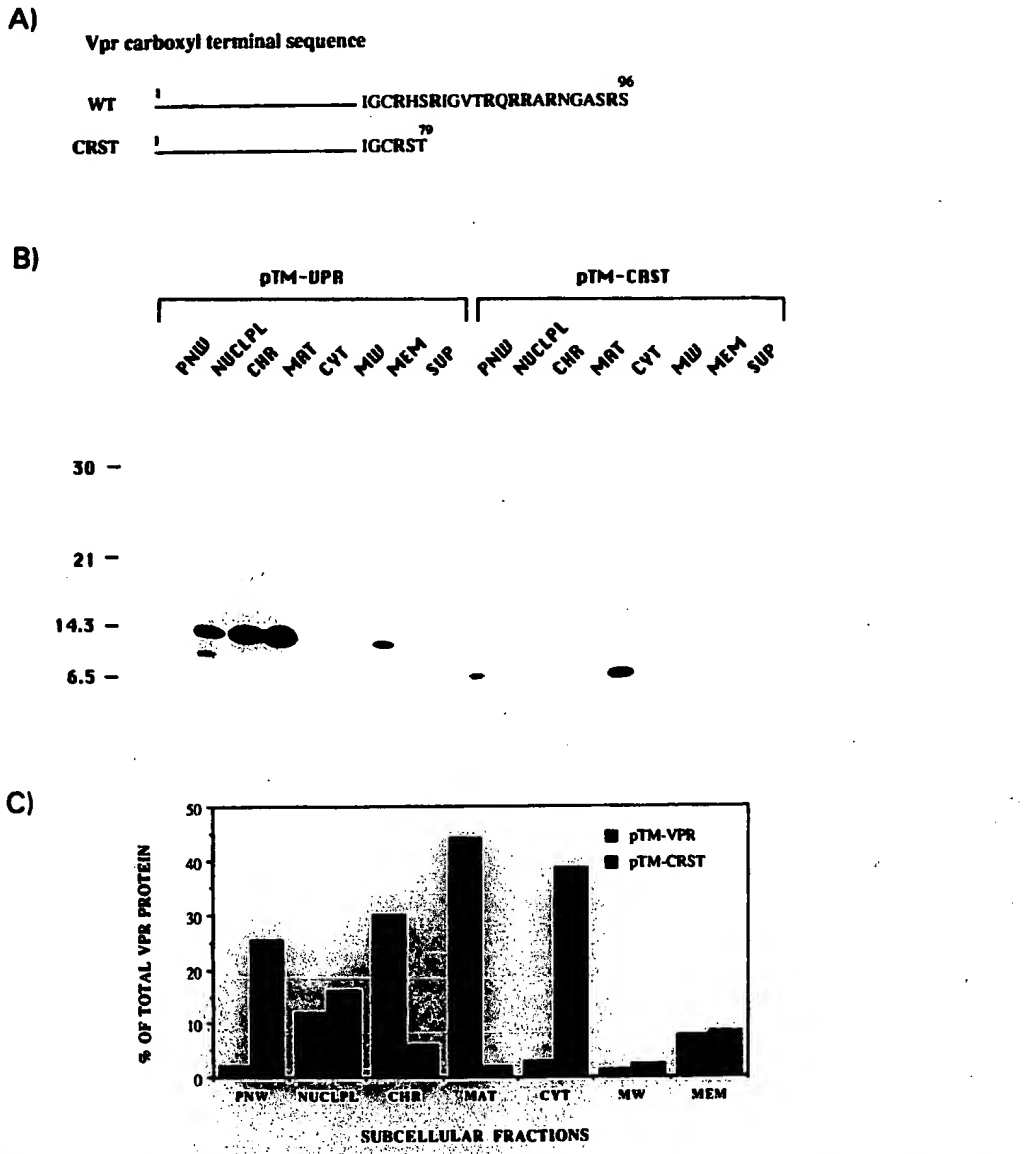


FIG. 3. Subcellular distribution of vaccinia virus-expressed parental Vpr and carboxyl-terminal truncation mutant CRST in BSC40 cells. (A) Schematic drawing of the Vpr protein, indicating the carboxyl-terminal arginine-rich sequence of the wild type (WT) and of the truncation mutant, CRST. (B) Vpr expressed from pTM3 in vTF7-3-infected cells. Cells were labeled with [$^3$H]leucine and fractionated into postnuclear wash (PNW), nucleoplasm (NUCLPL), chromatin (CHR), nuclear matrix (MAT), cytosol (CYT), membrane wash (MW), membranes (MEM), and cellular supernatant (SUP). Molecular mass markers are shown at the left in kilodaltons. (C) Proportion of VPR in each subcellular fraction as determined by laser densitometry from pTM-VPR (solid bars)- or pTM-CRST (hatched bars)-transfected cells.

preimmune serum or the anti-Vpr antibody (not shown). These smaller proteins may represent nonspecific cleavage products, products from initiation at a downstream AUG codon, or premature translational termination. No effects on Gag protein expression were noted with coexpression of Vpr.

Expression of pTM-VPR alone did not result in export in the cell supernatant (Fig. 5A, right). Expression of pTM-GAG resulted in the 55-kDa product in the cell supernatant. Coexpression of pTM-GAG with pTM-VPR promoted the export of Vpr into the cell supernatant.

To determine whether the viral proteins released into the cell supernatant were associated with particles, sucrose gradient analysis was performed (Fig. 6). Particles were first concentrated from the cell supernatant samples by centrifugation through a 20% sucrose cushion. The resultant particulate material was resuspended and analyzed on a linear 20 to 60% sucrose gradient. Each fraction was concentrated with 10% trichloroacetic acid and analyzed by SDS-PAGE. No particle-associated protein was found from cells transfected with pTM-VPR alone (Fig. 6A). Expression of pTM-GAG alone resulted in particle-associated Gag protein banding in fractions 10 and 11, at a density of 1.16 to 1.17 g/ml (Fig. 6B). Expression of pTM-GAG together with pTM-VPR resulted in cosedimentation of both Vpr and Gag in fractions 11 and 12, at a density of 1.16 to 1.17 g/ml (Fig. 6C).

Vpr packaging was also assessed with a clone expressing a

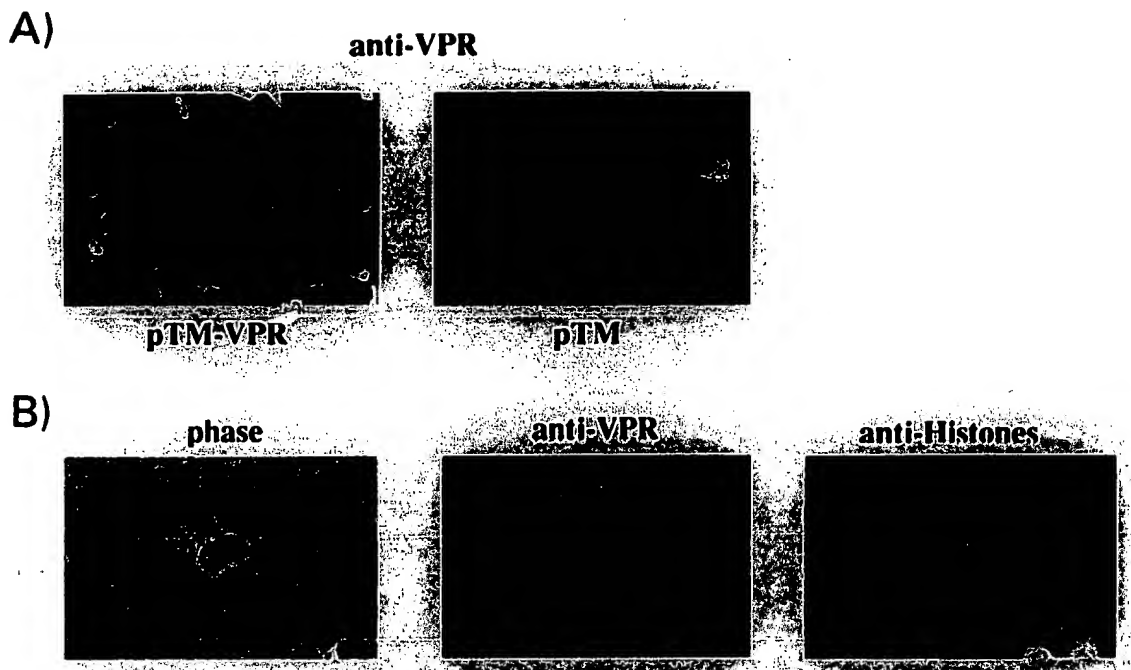


FIG. 4. Immunofluorescence localization of Vpr in BSC40 cells. (A) pTM-VPR (left)- or pTM (right)-transfected vTF7-3 infected cells were incubated with the anti-Vpr antiserum and an FITC-conjugated goat anti-rabbit immunoglobulin. Magnification, ×188. (B) Higher magnification (×752) by phase-contrast microscopy (left) and fluorescence microscopy (middle and right) of a representative cell incubated with anti-Vpr antiserum and FITC-conjugated goat anti-rabbit immunoglobulin (middle; filter with excitation range of 450 to 490 nm and emission range of 520 to 560 nm) and antihistone antiserum and rhodamine-conjugated goat anti-mouse immunoglobulin (right; filter with excitation range of 510 to 560 nm and emission range of >590 nm). Diffuse nuclear (thin arrow) and focal perinuclear staining (thick arrow) are indicated in the middle panel.

truncated form of the Gag precursor protein, pTM-GAG(p41), in which all of the amino acids following the first Cys-His box of NC as well as the C-terminal p6 coding sequence were removed. This construct has been shown to produce virus-like particles in the vaccinia virus infection-transfection system in a manner similar to that of pTM-GAG (not shown). When coexpressed with pTM-VPR, p41 appeared in the supernatant but did not result in the export of Vpr from the cells (Fig. 5B). The failure to detect Vpr in the cell supernatant was due to the absence of Vpr export rather than to the lower quantity of p41 Gag particles produced, since no Vpr was detected, even after overexposure of the autoradiogram shown in Fig. 5B. In contrast, in the same experiment, production of p55 from pTM-GAG resulted in significant export of Vpr.

## DISCUSSION

**Localization of Vpr in the nucleus.** In this study, we used three different expression systems to provide evidence for the localization of a significant proportion of Vpr in the nucleus, as demonstrated by subcellular fractionation techniques. In HIV-1-infected PBMCs, 26% of the expressed Vpr was found in the nucleus (Fig. 1). In contrast, when Vpr was expressed in the absence of other viral components by using an SV40 expression plasmid, almost all of the protein was found in the nucleus (Fig. 2). Similar results were obtained with the vaccinia virus expression system, in which case 84% of the Vpr was found in the nucleus (Fig. 3). Results of the indirect immunofluorescence experiments support the results obtained by using subcellular fractionation techniques, indicating nuclear staining in 66% of Vpr-expressing cells (Fig. 4; see Results).

Further fractionation of the isolated nuclei provides additional evidence for Vpr localization in the nucleus rather than in membranes loosely associated with the nuclear membranes. These experiments identified the predominant association of Vpr with the chromatin and nuclear matrix fractions (Fig. 2 and 3). The association of Vpr with the nuclear matrix is unlikely to be spurious, since it is resistant to Nonidet P-40, DNase, and high-salt extraction procedures. Although the role of the nuclear matrix in transcriptional regulation is unclear, several studies have indicated that it may play an important role. The nuclear matrix has been reported to have a role in mRNA transcription and processing via its involvement in attachment and/or association with newly transcribed mRNA (20), ribonucleoprotein particles (11), and pre-mRNA splicing machinery (36, 46). Several gene products, characterized for their ability to promote oncogenic transformation, are also associated with the nuclear matrix. These include the large T antigen of polyomavirus (4), *myc* gene products (9), the adenovirus E1A protein (10), and the Tax protein of HTLV-I (43). The presence of Vpr in the nuclear matrix might indicate a role in *trans* activation of viral gene expression or RNA processing. This is consistent with a report by Cohen and colleagues suggested that Vpr may serve as a *trans* activator of HIV-1 gene expression as well as a *trans* activator of other genes (7). However, the mechanism of this effect and its relevance to Vpr action during virus replication remain unclear. Alternatively, Vpr association with the nuclear matrix may affect host cell gene expression. This view is consistent with a recent report that Vpr induces muscle cell differentiation (24).

Though Vpr lacks a classical nuclear localization signal (15), the carboxyl-terminal portion of the protein is rich in basic amino acids. A truncation mutation which removes the carboxyl-terminal 19 amino acids was found to impair Vpr localiza-

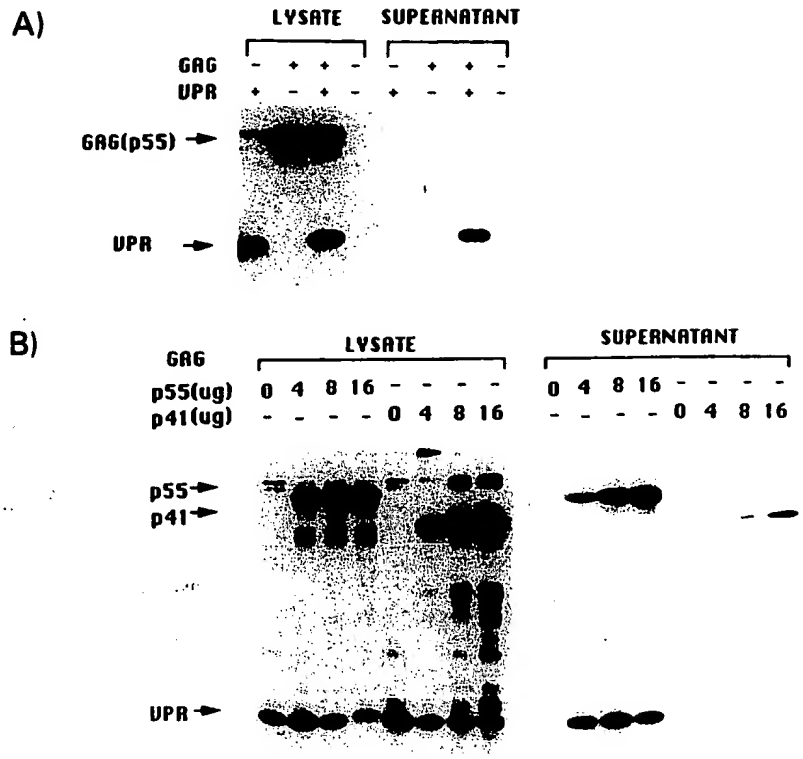


FIG. 5. Influence of Gag expression on Vpr localization. (A) BSC40 cells ($10^6$) were grown on 60-mm-diameter plates overnight and then infected with vTF7-3 and transfected with 7.5 μg of pTM-VPR (VPR) and/or 7.5 μg of pTM-GAG (GAG). The negative control cells were transfected with 15 μg of pTM vector. The cells were labeled with 100 μCi of [$^3$H]leucine per ml for 16 h, scraped in PBS, and resuspended in RIPA buffer. The cell lysates and cell supernatant fractions were immunoprecipitated with both the anti-Vpr and anti-Gag antisera and analyzed by SDS-PAGE. The electrophoretic positions of Gag and Vpr are shown at the left. (B) BSC40 cells ($0.3 \times 10^6$) on 35-mm-diameter plates were infected and transfected with 2 μg of pTM-VPR and 0, 4, 8, or 16 μg of pTM-GAG(p55) or pTM-GAG(p41), as indicated at the top. The cells were labeled and analyzed as described above.

tion in the nucleus (Fig. 3). Furthermore, the distribution of the small proportion of truncated Vpr found in the nucleus was distinctly different from that of full-length Vpr, with the truncated Vpr localized predominantly in the nucleoplasm and very little Vpr in the nuclear matrix or chromatin fractions. It is possible that truncation of the carboxyl-terminal portion of Vpr alters the conformation of the molecule. Alternatively, it is possible that the carboxyl-terminal arginine-rich sequence serves as at least part of a nuclear localization signal. This view is supported by our preliminary observations that attachment of the C-terminal 19-amino-acid Vpr sequence onto β-galactosidase directs this protein to the nucleus (not shown).

Previous studies with lymphoid cells had indicated an important functional role for the C-terminal Vpr sequence (29). Therefore, these findings are consistent with an important role for Vpr localization in the nucleus for HIV-1 replication.

**Membrane-associated Vpr.** Though very little Vpr could be identified in the cytosol, a small proportion was consistently associated with the membrane fraction. This observation is in agreement with findings of Sato and colleagues (34). In HIV-1-infected PBMCs, 20% of Vpr was found in the membrane fraction (Fig. 1), whereas with the vaccinia virus expression form of the protein, 8% was found in the membrane (Fig. 3B and C). The indirect immunofluorescence experiments also suggested that some Vpr is found at an extranuclear site but closely associated with the nucleus (Fig. 4; see Results). The latter site may represent intracellular membranes, possibly with either the endoplasmic reticulum or Golgi apparatus. However, a Golgi location for Vpr is unlikely, since brefeldin A treatment did not change Vpr localization (not shown). The nature and significance of membrane localization of VPR require further analysis.

**VPR export from cells and incorporation into virus particles.** Several previous studies have demonstrated that HIV- and SIV-expressed Vpr is incorporated into virus particles (6, 44, 45). This finding is in agreement with our observation that 50% of Vpr expressed in HIV-1-infected PBMCs is exported from the cells (Fig. 1). Vpr expression in the absence of other viral components resulted in no detectable export (Fig. 3 and 5). However, coexpression with the Gag p55 precursor protein resulted in export of VPR from the transfected cells (Fig. 5) and incorporation into virus-like particles (Fig. 6). Thus, Vpr incorporation into virus particles is independent of viral envelope incorporation. This finding suggests that Vpr associates directly or indirectly with a portion of the Gag precursor protein. The finding that the p41 truncation form of Gag is unable to package Vpr suggests the possibility of an interaction between either the p9 nucleocapsid protein or the proline-rich p6 protein and Vpr.

Although the significance of Vpr incorporation into virions

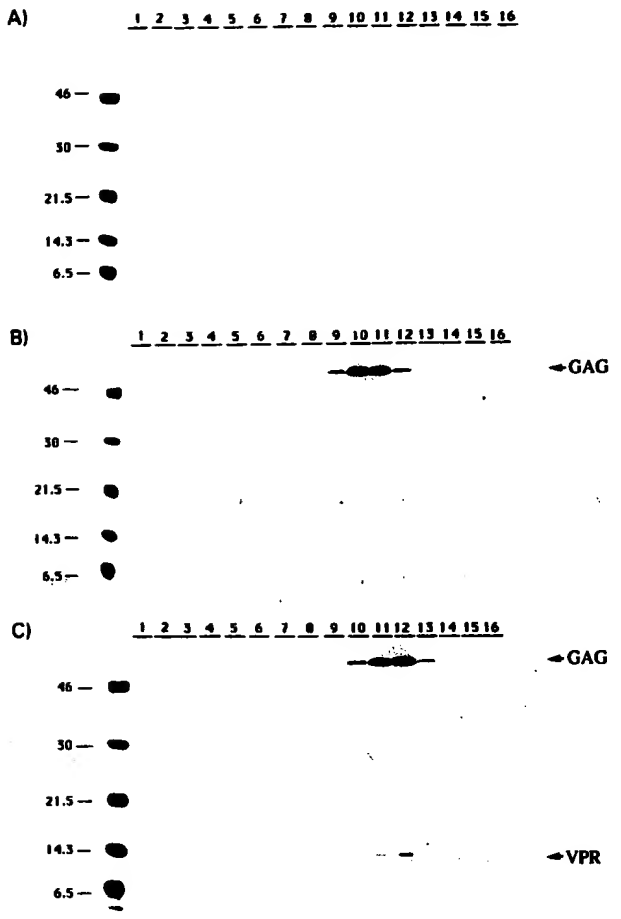


FIG. 6. Sucrose gradient analysis of particles released from BSC40 cells transfected with pTM-VPR (A), pTM-GAG (B), pTM-VPR and pTM-GAG (C). Fractions were precipitated with 10% (wt/vol) trichloroacetic acid and analyzed by SDS-PAGE. Fraction 1 is from the bottom and fraction 16 from the top of each gradient. Molecular mass markers are shown at the left in kilodaltons.

is unclear, it is likely that this protein plays an important role in early events in the virus life cycle. It is tempting to speculate that the nuclear localization domain of Vpr allows targeting of the viral preintegration complex to the nucleus. Further studies on this important regulatory protein will be required to fully elucidate its role in the HIV life cycle.

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# Human Immunodeficiency Virus *vpr* Gene Encodes a Virion-Associated Protein

XIN YUAN, ZENE MATSUDA, MICHIKO MATSUDA, MAX ESSEX, and TUN-HOU LEE

## ABSTRACT

**The *vpr* gene of human immunodeficiency virus type 1 (HIV-1) is one of the seven accessory genes that are believed to have roles in the virus replication cycle. We report here the detection of a 13 kD *vpr* protein in sucrose gradient-purified HIV-1. This protein was not detected in cells infected with a virus having a truncated *vpr* gene that lacks the potential to encode for 26 C-terminal amino acid residues. These findings raise the possibility that virion-associated *vpr* proteins may be involved in the early life cycle of HIV-1 replication and suggest that the C-terminal region of the *vpr* gene is essential for its expression.**

## INTRODUCTION

IN ADDITION TO *gag*, *pol*, AND *env* GENES, which are commonly found in the genome of replication-competent animal retroviruses, seven other accessory genes have been identified in human immunodeficiency virus type 1 (HIV-1). These accessory genes include *vpr*,[1] *vif*,[2–4] *vpu*,[5–7] *tat*,[8–10] *rev*,[11,12] *tnv*/*tev*,[13,14] and *nef*.[15,16] The *vpr* gene of HIV-1 was initially identified as an open reading frame (ORF) in molecularly cloned proviruses.[17] This ORF is highly conserved among different HIV-1 isolates and shares substantial homology with the analogous orf found in the genomes of HIV-2 and simian immunodeficiency virus (SIV).[17–19]

The first proof that the *vpr* ORF is indeed a coding sequence of HIV-1 came from the observation that a recombinant protein encoded by the *vpr* orf was found to react with sera from some HIV-1-infected individuals.[17] Two subsequent studies suggested that the *vpr* gene might have a role in the upregulation of HIV-1 expression.[20,21] One of these demonstrated that premature terminations introduced into the *vpr* gene caused delay in virus replication and reduction in cell killing.[20] The other study showed that the *vpr* gene had a stimulatory effect on the HIV-1 long terminal repeat (LTR) and several heterologous promoters.[21]

The *vpr* protein detected in HIV-1-infected cells was identified as a 15 kD protein and thought to have a role in boosting HIV production in the later part of the HIV-1 life cycle when expression of the *tat* protein decreases.[21] However, a potential role for the *vpr* protein in the early part of the virus life cycle cannot be excluded if the *vpr* protein is found to be present in the virions. This study addresses the possibility that the *vpr* gene of HIV-1, like one of the accessory genes of HIV-2/SIV,[22–24] encodes a virion-associated protein.

## MATERIALS AND METHODS

### *Plasmid*

The plasmid pRTUB is a subclone of an HIV-1 provirus clone, BH10.[15] This plasmid is identical to the previously described plasmid, pRTUA,[5] except that the 2.2 kb KpnI–KpnI fragment derived from the BH10 was in the opposite orientation. A 979 bp HaeIII-HaeIII fragment of pRTUB, which contained most of the *vpr* coding sequence, was cloned into a previously described expression vector, p806[25] at the SmaI site, to generate p806R. Both BH10 and HXB2 share the same protein sequence in the *vpr* ORF.[26] The *vpr* ORF in the p806R was verified by the Sanger DNA sequencing method[27] to be in-frame with the preceding v-H*ras*.

### Sera

The procedures for the expression and partial purification of the recombinant *vpr* protein expressed by p806R was as described previously.[5,22] After further separation of the partially

Department of Cancer Biology, Harvard University School of Public Health, Boston, MA 02115.

purified recombinant vpr protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the *vpr* protein was excised and used to raise a heterologous goat antiserum.[5,22] The goat was boosted with the recombinant protein weekly between weeks 2 and 6 after initial immunization. The serum from the 7-week bleeding were used in this study. Serum samples of HIV-1-infected or uninfected subjects were from the Fenway Community Health Center in Boston, Walter Reed Army Institute of Research, and Harvard School of Public Health.

### *Oligonucleotide-directed mutagenesis*

To generate the molecular provirus clone HXB2R, thymidine 5351 (numbered according to Ref. 28) in the *vpr* orf of HXB2 was removed by site-directed mutagenesis. Briefly, a 1.3 kbNdeI-NdeI fragment from HXB2, which contained the *vpr* orf, was cloned into the NdeI site of phagemid pGEM-3Zf(−)D, which lacks the polylinker sequence of pGEM-3Zf (−) (Promega Corp., Madison, WI). Single-stranded uracil-containing phage DNA was isolated and used as the template for oligonucleotide-directed mutagenesis using previously described procedures.[29] The mutagenic oligonucleotide, 5′-CCCAATTCTGAAATG-GATAAACAGC-3′, was synthesized on a Milligen/Biosearch 8750 DNA synthesizer. The 1.3 kb NdeI-NdeI fragment containing the desired deletion was verified by DNA sequencing and ligated to the NdeI-digested HXB2 fragment of approximately 14 kb to generate the HIV-1 molecular clone, HXB2R.

### *Cells and viruses*

Human T-cell line JM[30] was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (complete medium). HIV-1 HXB2 and HXB2R viruses were harvested from culture supernatants of COS-1 transfectants 48 h posttransfection. The transfection of COS-1 cells was carried out by DEAE/dextran method.[22] The culture supernatants were filtered through 0.45 μm filters before the amount of virus was determined by the reverse transcriptase (RT) assay.[31]

### *Radioimmunoprecipitation*

Two million JM cells in 9 ml complete medium were infected with 1 ml of cell-free supernatant from COS-1 transfectants for 12 h before the cells were washed with RPMI-1640 and resuspended in 10 ml complete medium. Based on RT assay, the virus content in the inoculum was 100,000 cpm/ml. The RT level of the HXB2R- or HXB2-infected JM cultures was followed during the course of infection, and the infected cells were metabolically labelled with 100 μCi/ml [$^{35}$S]cysteine (NEN, Boston, MA; 300 TBq/mmol) or [$^{3}$H]leucine (Amersham Corp. Arlington Heights, IL; 4.4–7.0 TBq/mmol) for 12 h at Day 10 or Day 11 postinfection when RT reached its peak. At the end of labeling, the cells were centrifuged at 1,000 g for 5 min, washed once with cold phosphate-buffered saline (PBS), and lysed in 3 ml radioimmunoprecipitation assay (RIPA) lysis buffer as described previously.[2] The culture supernatants were filtered through 0.45 μm filters to remove cell debris and centrifuged in a Beckman SW28 Rotor at 20,000 rpm for 2 h through a 20% (w/v) sucrose cushion. The virus pellet was subsequently resuspended in 0.5 ml RPMI-1640 and subjected to further purification through a discontinuous sucrose gradient. The discontinuous sucrose gradient was prepared by overlaying eleven 3 ml layers of sucrose sequentially with the following concentrations: 55% (w/v), 50%, 45%, 42.5%, 40%, 37.5%, 35%, 32.5%, 30%, 25%, and 20%. The virus overlaid on top of this gradient was centrifuged in a Beckman SW28 rotor for 20 h before 12 fractions of 3 ml each were collected dropwise from the bottom of the centrifuge tube. The amount of radiolabeled virus in each fraction was measured by scintillation count of a 2 μl aliquot from each fraction. The fraction containing the highest radioactivity was pooled with two neighboring fractions, diluted threefold with PBS, and centrifuged in a Beckman 70Ti rotor for 2 h at 35,000 rpm. The virus pellets were resuspended in 400 μl RIPA lysis buffer. In the parallel analyses using nonradiolabeled viruses, the fraction that contained the highest RT activity coincided with the fraction having the highest radioactivity. Aliquots of 100 μl radiolabeled cell lysates or virus lysates were immunoprecipitated with 20 μl goat anti-*vpr* sera and 10 μl human HIV-1 seropositive or negative sera. The immunoprecipitates were analyzed as described before.[2]

## RESULTS

### *Expression of recombinant* vpr *protein*

Figure 1a shows the bacterial expression vector, p806R. The *vpr* recombinant protein expressed by this vector contains 70 amino acid residues from the C-terminus of *vpr* ORF of BH10. The *vpr* coding sequence is preceded by 111 N-terminal amino acid residues of v-H*ras* and 6 amino acid residues from the polylinker sequence (Fig. 1a). The expression of this fusion protein is under the control of the inducible *tac* promoter. After induction with isopropyl-β-D-thiogalactopyranoside (IPTG), a protein of approximately 25 kD was detected in the induced culture of bacteria bearing the plasmid, p806R (Fig. 1b). This protein was not detected in the uninduced culture (Fig. 1b). By immunoblotting analysis, this recombinant protein was reactive with a goat anti-*ras* antibody,[22,25] 12 of the 46 HIV-1 seropositive sera and none of the 10 HIV-1 seronegative sera analyzed (data not shown). This is in agreement with the results of DNA-sequencing analysis which indicate the coding sequence of the *vpr* ORF is in-frame with the preceding sequences. The recombinant *vpr* protein purified on SDS-PAGE (Fig. 1b) was used to raise a goat anti-*vpr* serum for identifying the *vpr* protein.

To identify the *vpr* gene product, we studied JM cells infected by HIV-1 molecular clones HXB2R and HXB2. The *vpr* ORF of the HXB2R, like that of HIV-$1_{BRU}$ and HIV-$1_{MN}$, has the potential to encode a *vpr* protein of 96 amino acid residues. As shown in Figure 2a, the 26 C-terminal amino acid residues of HXB2R *vpr* are nearly identical to those in the *vpr* orf of HIV-$1_{BRU}$ and HIV-$1_{MN}$. In contrast, the *vpr* ORF in the HXB2 clone has the potential to encode for vpr protein of only 78 amino acid residues because of the extra thymidine at position 5351 (Fig. 2b). Some of the C-terminal amino acid residues that are highly conserved among HIV-1 and the more distantly related HIV-2 and SIV (Fig. 2a) are not present in this pretruncated version of *vpr* protein.

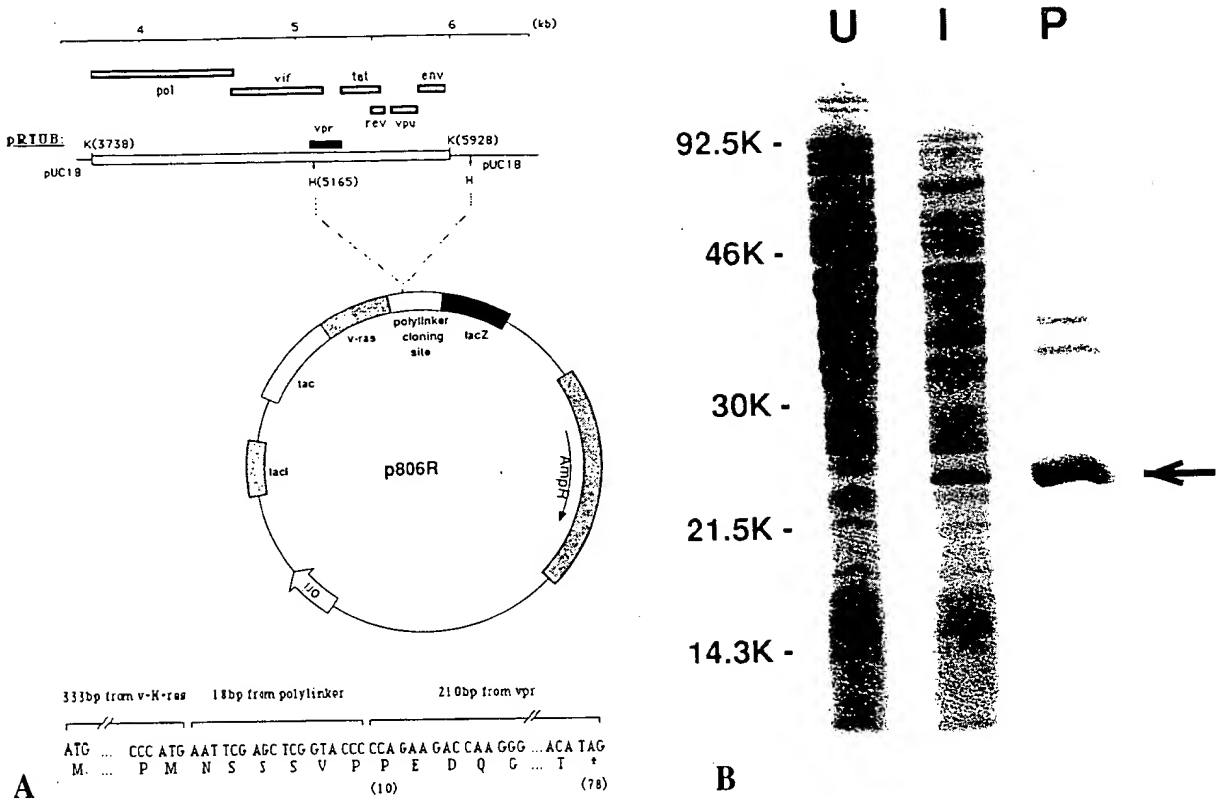
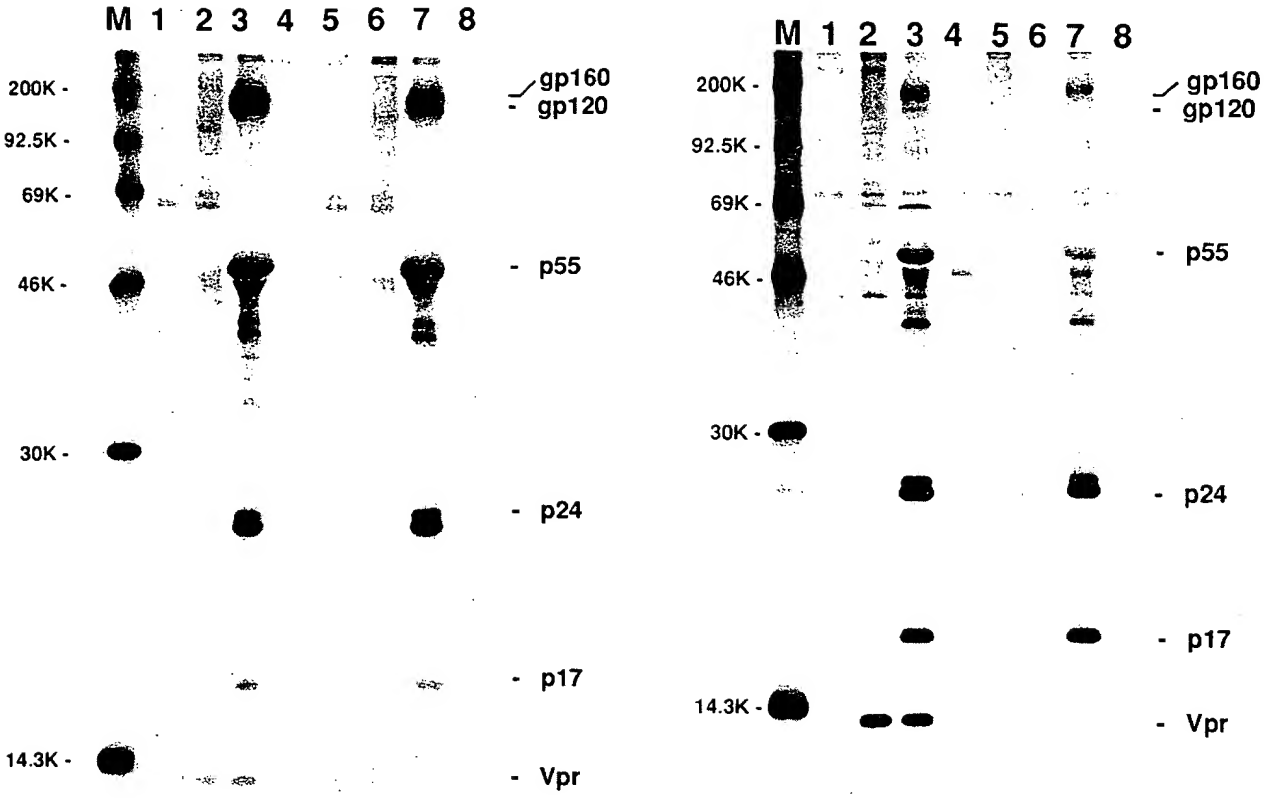



**FIG. 1.** Expression of recombinant *vpr* protein. (a) Bacterial expression vector, p806R, was constructed as described in Materials and Methods. The insert cloned into the p806 vector and its relationship with the HIV-1 genes are depicted in the top panel. The bottom panel shows the predicted amino acid sequence (abbreviated to single letter) of the *vpr* recombinant protein. The number beneath the amino acid sequence indicates the position of the amino acid residue in the *vpr* orf. kb = kilobases, K = KpnI, H = HaeIII, AmpR = ampicillin resistance gene, *lac*I = repressor gene for *lac* promoter, *tac* = *tac* promoter, v-*ras* = v-H*ras* gene, *lac*Z = B-galactosidase gene. (b) SDS-PAGE profile of *vpr* recombinant protein. Whole cell lysates from IPTG-induced (lane I) and uninduced (lane U) p806R-bearing bacteria, as well as partially purified recombinant protein (lane P) are shown. The arrow indicates the position of the *vpr* protein. Migration of protein $M_r$ standards is shown on the left in kilodaltons (K).

The cell lysates from HXB2R-infected JM cells which were metabolically labeled with [$^{35}$S]cysteine were subjected to immunoprecipitation analysis (Fig. 3a). A 13 kD protein was precipitated by the postimmune goat anti-*vpr* antiserum and sera of HIV-1-infected donors from the lysates of HXB2R-infected cells, but not by the preimmune and sera from HIV-1 seronegative donors (Fig. 3a). In contrast, the 13 kD protein was not detected by any of the sera studied from cells infected by HXB2 which has a prematurely truncated *vpr* ORF containing no cysteine residues. These observations strongly suggest that the 13 kD protein is the product of the *vpr* gene.

To study whether a pretruncated version of *vpr* protein can be detected in HXB2-infected cells, radioimmunoprecipitation analysis was performed using antigens prepared from HXB2-infected cells metabolically labeled with [$^3$H]leucine. As shown in Figure 3b, the 13 kD protein was detected in HXB2R-infected


**FIG. 2.** C-terminal region of *vpr* protein. (a) Alignment of the C-terminal region of *vpr* protein of HXB2R with those of several HIV-1 and SIV isolates. The dashes indicate the identical residues among these virus clones. Asterisk = stop codon. The spaces in the HIV-1 sequences are for the purpose of alignment. (b) Comparison of vpr sequences between the HXB2 and HXB2R. The underlined thymidine residue was deleted from HXB2 as described in materials and methods.




A B

**FIG. 3.** (a) Radioimmunoprecipitation of HIV-1-infected cells labeled with [$^{35}$S]cysteine. Lysates of JM cells infected by HXB2R (lanes 1–4) and HXB2 (lanes 5–8) were immunoprecipitated with preimmune goat antiserum (lanes 1 and 5), goat postimmune anti-*vpr* antiserum (lanes 2 and 6), a representative HIV-1 seropositive human serum (lanes 3 and 7), and HIV-1 seronegative human serum (lanes 4 and 8). The major viral gene products are indicated on the right of the figure. K = kilodalton, M = molecular size marker. (b) Detection of *vpr* protein in HIV-1-infected cells labeled with [$^{3}$H]leucine. JM cells infected with HXB2R (lanes 1–4) or HXB2 (lanes 5–8) were labeled with [$^{3}$H]leucine. Cell lysates were immunoprecipitated with preimmune (lanes 1 and 5) and postimmune (lanes 2 and 6) goat anti-*vpr* antisera; with a representative HIV-1 seropositive human serum (lanes 3 and 7) and HIV-1 seronegative human serum (lanes 4 and 8).

cells using the postimmune goat anti-*vpr* antiserum and sera of HIV-1-infected donors. However, no truncated version of the *vpr* protein was detected in HXB2-infected cells.

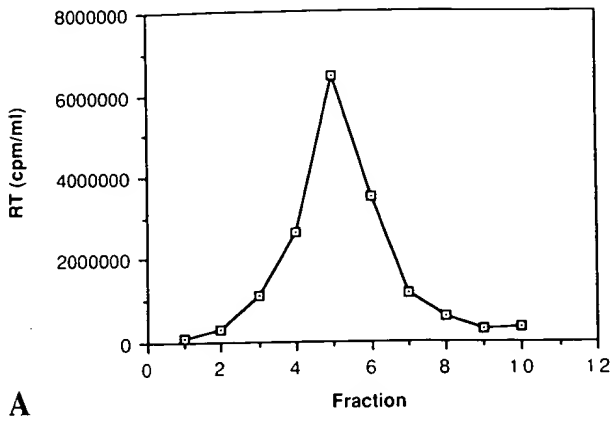
### *Virion-associated* vpr *protein*

Figure 4a shows the distribution of RT activity of sucrose gradient-purified virions from HXB2R-infected cells. The peak RT activity typically falls in fraction 5 corresponding to approximately 40% (w/v) sucrose. Cell-free virions from [$^{35}$S]cysteine-labeled HXB2R-infected culture were purified in sucrose gradients prepared in parallel experiments and subjected to radioimmunoprecipitation analysis. As shown in Figure 4b, a 13 kD protein was precipitated by the postimmune goat anti-*vpr* serum and sera from HIV-1-infected donors, but not by the preimmune goat serum or sera from HIV-1 seronegative donors. While the *gag*, *pol*, and *env* encoded proteins were detected in viral lysates similarly prepared from HXB2-infected culture, no 13 kD *vpr* protein was detectable.

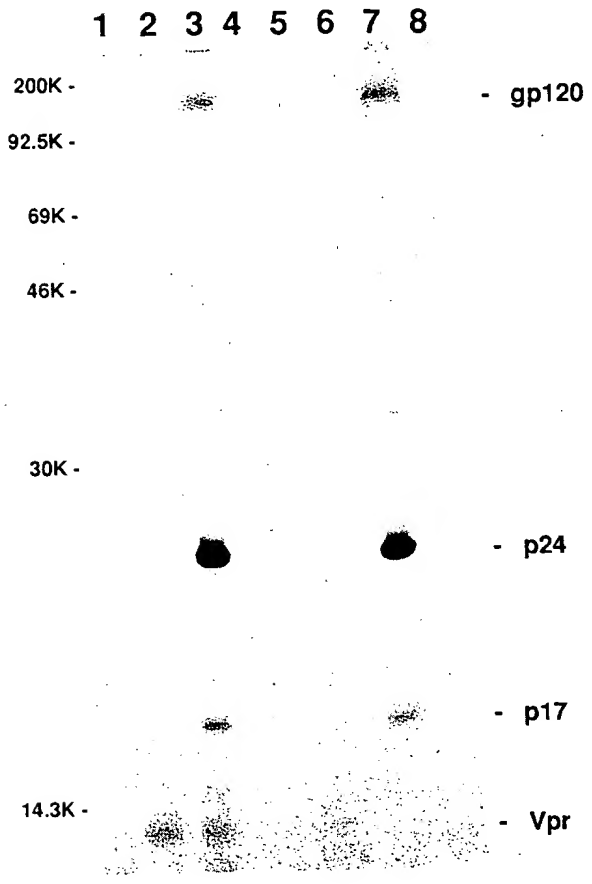
## DISCUSSION

Evidence was provided here to show that, in addition to proteins encoded by the *gag*, *pol*, and *env* genes, HIV-1 virions contain another protein encoded by *vpr*, one of the seven accessory genes. This protein could not be detected in cells infected by a virus with a truncated *vpr* gene. This result is in contrast with a previous report based on in vitro translation[21] and can possibly be explained by the loss of antigenicity associated with the truncation. However, because of the polyclonal nature of the goat anti-*vpr* serum, which was raised against a recombinant protein containing most of the N-terminal residues of *vpr*, a more plausible interpretation of this finding is that the C-terminal region of the *vpr* gene is critical for its expression. This result could theoretically be caused by the reduction in the stability of the protein product, the efficiency of protein translation, and/or, albeit less likely, mRNA stability.

A possibility for this virion-associated protein is that it is a structural component of the virion. Nonetheless, because the




A




B

**FIG. 4.** Sucrose gradient purification and radioimmunoprecipitation of [$^{35}$S]cysteine label HIV-1 virions. (a) RT activity of sucrose gradient-purified HXB2R virions. Fraction one corresponds to the bottom of the gradient. (b) Fractions 4, 5, and 6 of each gradient were pooled and HXB2R (lanes 1–4) and HXB2 (lanes 5–8) viruses were pelleted. Viral pellets were then subjected to immunoprecipitation with goat preimmune (lanes 1 and 5), postimmune anti-*vpr* antiserum (lanes 2 and 6), a representative HIV-1 seropositive human serum (lanes 3 and 7), and HIV-1 seronegative human serum (lanes 4 and 8).

infectivity of several *vpr* mutants, such as HXB2,[17] $HX_{BRU}$,[21] pR2[32], and pNL-Af2,[20] was known to be unimpaired,[17,20,21,32] it is unlikely that such a dispensable virus protein represents an integral part of the HIV-1 structure.

The *vpr* protein was reported to have a stimulatory effect on HIV-1 LTR and several heterologous promoters.[21] The inability to identify specific sequences in the HIV-1 LTR, which mediates the observed function of the *vpr* gene,[21] raises the possibility that the virion-associated *vpr* protein may have a function similar to that of herpes simplex virus (HSV) VP16.[33,34] This virion-associated protein in HSV is known to work in concert with a cellular factor to upregulate the expression of immediate early HSV genes in the early part of the virus life cycle.[35] Interactions between *vpr* proteins and cellular factors may help to establish HIV-1 infection in resting cells or in some cell types in vivo.

It is worth noting that the C-terminal region of the *vpr* protein is rich in arginine residues (Fig. 2a). The arginine-rich region of HIV-1 *tat* and *rev* has been demonstrated to have a role in their nuclear localization.[36–43] This raises the possibility that *vpr* may also be localized in the nucleus of infected cells. However, arginine-rich sequences have also been found in several potential RNA-binding proteins, including ribosomal proteins, some RNA viral capsid proteins and the lambda phage N protein.[44] The possibility that the virion-associated *vpr* protein may exert its function by directly binding to genomic RNA, the newly synthesized viral transcripts, and/or cellular transcripts, remains to be considered.

## RECENT DEVELOPMENT

Since this manuscript was submitted, a similar finding has been reported by Cohen et al.[45]

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Address reprint requests to:
*Dr. Tun-Hou Lee*
*Department of Cancer Biology*
*Harvard University School of Public Health*
*665 Huntington Avenue*
*Boston, MA 02115*

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# Induction of Neutralizing Antibodies Against Human Immunodeficiency Virus Type 1 Using Synthetic Peptide Constructs Containing an Immunodominant T-Helper Cell Determinant from vpr

*Pablo Sarobe, *Juan-José Lasarte, *José-Javier Golvano, *Isidro Prieto, †Arturo Gullón, ‡María-José Soto, ‡Pablo Labarga, *Jesús Prieto, and *Francisco Borrás-Cuesta

**Departamento de Medicina Interna and †Departamento de Genética, Universidad de Navarra, Pamplona; and ‡Departamento de Medicina Interna, Hospital San Millán, Logroño, Spain*

**Summary:** Identification of immunodominant T-helper-cell determinants after natural infection is an important step in the design of immunogens for potential use in vaccination. Using cells from human immunodeficiency virus type 1 (HIV-1)–infected individuals and a panel of peptides encompassing the sequence of the regulatory protein vpr from HIV-1, we identified the T-helper determinant QLLFIHFRIGCRHSR, which is active in 37.5% of these individuals. To gain insight on the efficacy of this peptide in helping induce neutralizing antibodies against a B-cell determinant (BD), we synthesized constructs containing B- and T-cell determinants and tested them in BALB/c mice, the highest responders to the T-cell determinant moiety among several strains tested. These immunogens induced antibodies against two chosen B-cell determinants from HIV-$1_{IIIB}$ gp160 (amino acids 310–322 from the V3 loop of gp120 and 736-751 from gp41) that were able to neutralize HIV-1 infection *in vitro*. The highest neutralization titer against HIV-$1_{IIIB}$ was obtained by immunization with the homopolymer of the construct containing the T-cell epitope from vpr and the B-cell epitope from the V3 loop. We believe that the immunodominant T-cell determinant from vpr is a promising epitope to consider in the design of future peptide vaccines. **Key Words:** Human immunodeficiency virus type 1—vpr—T-cell determinant—B-cell determinant—Immunogenic constructs—gp120 V3 loop—gp41.

It is well established that immunization with a T-helper-cell determinant ($TD_h$) covalently linked to a B-cell determinant (BD) can induce high antibody titers against the BD (1–4). This finding suggests the possibility of inducing antibodies against selected BDs of interest, a strategy that may prove useful to protect against human immunodeficiency virus type 1 (HIV-1) infection because it would avoid the appearance of antibodies against certain BDs of the envelope protein of HIV-1 that are induced with this protein and that are known to enhance viral infectivity (5,6).

Because memory T-helper cells are required to elicit a secondary response against an antigen (7), the $TD_h$ moiety (or moieties) of immunogenic peptide constructs potentially useful in vaccination

Address correspondence and reprint requests to F. Borrás-Cuesta at Universidad de Navarra, Facultad de Medicina, Apartado 273, 31008 Pamplona, Spain.

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against HIV-1 should belong to the antigens of this virus. Among them, the regulatory protein vpr is of special interest because it is one of the most highly conserved antigens present in the virions of all viral mutants (8). Thus, with the aim of developing immunogens of the type $TD_h$-BD, we identified the regions of vpr that were most commonly recognized by T-helper lymphocytes of HIV-1-infected individuals. We show herein that the sequence QLLFIHFRIGCRHSR is an immunodominant $TD_h$ from vpr. Moreover, its coupling to a peptide from the V3 loop of HIV-$1_{IIIB}$ gp120 creates an immunogen that elicits antibodies specific to the loop that are able to neutralize HIV-$1_{IIIB}$ infection *in vitro*.

## MATERIALS AND METHODS

### Mice

Female BALB/c, CBA, and C57BL/6 mice, 4–8 weeks old, were purchased from Panlab (Barcelona, Spain).

### Peptide Synthesis and Preparation of Homopolymers

Peptide immunogens were synthesized by the solid-phase method of Merrifield (9) using the $F_{moc}$ alternative (10). The synthesis was done manually, and the ninhydrin test of Kaiser et al. (11) was used to monitor every step. Couplings were repeated if necessary until a negative ninhydrin test was attained. All peptides were purified by high performance liquid chromatography on a reverse-phase C18 column. Peptide homopolymers were prepared from side-chain protected peptide monomers using carbodiimide, as has been described (12). Peptides encompassing the entire sequence of vpr and variant peptides from the V3 loop were synthesized with a multiple solid-phase peptide synthesizer (13).

### Proliferation Assays in Humans

For the assay of antigen-induced T-cell proliferation, peripheral blood lymphocytes from HIV-1-seropositive donors and controls were separated on lymphocyte separation medium (Lymphoprep, Pharmacia), washed three times, counted, and resuspended at $2 \times 10^6$ cells/ml in complete medium (RPMI 1640, 10% pooled human AB serum, and 1% gentamicin).

Cells (0.1 ml/well) were added in triplicate wells in a 96-well flat-bottomed plate and cultured without stimulation or stimulated with peptide at a final concentration of 15 μg/ml for 7 days. Cells were pulsed with 1 μCi/well for the last 18 h of culture and then harvested. The [$^3$H]thymidine incorporated was counted in a β-counter.

### IL-2 Assay in Mice

Eight-week-old mice were immunized at the base of the tail and footpads with 200 μl of an emulsion of peptide (100 μg/mouse) in complete Freund's adjuvant (1:1). Ten days after the primary injection, animals were killed and lymph node cells were removed. The lymphocytes were then plated on 96-well plates at $5 \times 10^5$ cells/well with RPMI-supplemented medium (RPMI supplemented with 10% fetal calf serum, 10 m*M* glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and $5 \times 10^{-5}$ *M* 2-mercaptoethanol) or serial dilutions of peptides in a 0.2-ml final volume. Supernatants (50 μl) were removed 24 h later and assayed for the interleukin-2 (IL-2) content, as already described (14).

### Immunization Experiments for Antibody Induction

Groups of three 4-week-old BALB/c mice were immunized by i.p. injection of 200 μl of an emulsion obtained by sonication of 100 μl of complete Freund's adjuvant and 100 μl of saline solution containing 60 μg of peptide antigen: they were boosted at days 30 and 45 with the same dose of antigen in incomplete Freund's adjuvant. Mice were eye-bled on days 30, 45, and 60 after the first injection.

### Titration of Antibodies

Antibodies were titrated by enzyme-linked immunosorbent assay (ELISA). Microtiter wells were coated by overnight incubation at 4°C with 50 μl of peptide solution (20 μg/ml of peptide in 0.1 *M* sodium carbonate buffer). Wells were then washed three times with a solution of phosphate-buffered saline (PBS). To block nonspecific antibody binding, the wells were incubated with this buffer, also containing 1% powdered milk and 0.1% Tween 20 (PBSMT), for 1 h at room temperature. After removing the PBSMT, 100 μl of different serum dilutions in PBSMT were added and incubated at 37°C for 1 h. Wells were washed three times with PBS and then incubated at 37°C for 1 h with a 1:1,000 solution of goat anti-mouse IgG biotinylated whole antibody (Amersham, England) in PBSMT. After washing three times with PBS, wells were incubated with a 1:500 dilution of horseradish peroxidase–streptavidin in PBSMT (Amersham) at 37°C for 1 h. After washing three times with PBS, the color reaction was started by adding 100 μl of a solution prepared by mixing 10 ml of 0.6% acetic acid (pH 4.7), 5 μl of 33% (w/v) hydrogen peroxide, and 100 μl of a 40 m*M* water solution of ABTS (2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid). After 60 min the plates were read at 405 nm in a Titertek Multiscan MKII (Flow Laboratories).

### HIV-1 Neutralizing Assay

The inhibition of HIV-1 infectivity was assayed using HT4LacZ-1 cells (a gift of Dr. Saragosti). The cells (10,000 cells/well) were added to a 96-well flat-bottomed plate and cultured at 37°C and 5% $CO_2$ for 18 h. Then, 100 μl of a mixture previously incubated at 37°C for 1 h, containing 50 μl of serum to be tested and 50 μl of diluted viral supernatant from Molt-3/HIV-$1_{IIIB}$ cell cultures (150–200 syncytia forming units), was added and cultured for 3 days. The plate was then developed and the syncytia counted as previously described (15). A group of three or more blue-stained nuclei was considered to be one syncytium.

## RESULTS

### Identification of $TD_h$ from vpr in HIV-1-Infected Patients

Peripheral blood mononuclear cells from 16 HIV-1-seropositive patients, with >500 $CD4^+$ cells/$mm^3$, were tested in a proliferative assay against 15-mer overlapping peptides (Table 1) encompassing the entire sequence of vpr. As shown in Table 2, only peptides vpr10, vpr17 and vpr20 were recognized by the cells. This last peptide was positive for six of the 16 HIV-1-seropositive patients tested (37.5%), but negative against a panel of 10 HIV-1-seronegative individuals.

### Study of the $TD_h$ Character of vpr20 in Different Mouse Strains

The potential $TD_h$ character of vpr20 in BALB/c ($H\text{-}2^d$), CBA ($H\text{-}2^k$), and C57BL/6 ($H\text{-}2^b$) mice was studied by immunization with this peptide and by measuring the IL-2 production after in vitro restimulation of their lymph node cells. Figure 1 shows that a higher stimulation index for IL-2 production is observed in BALB/c mice, proving that vpr20 is more immunogenic in BALB/c than in CBA or C57BL/6 strains.

### Induction of Antibodies in BALB/c Mice with Peptide Constructs Containing vpr20

To study the effect of vpr20 on the induction of antibodies against two BDs from gp160 of HIV-$1_{IIIB}$, the peptides shown in Table 3 were synthesized. BD1 represents residues 310–322 from the V3 loop of gp120, and BD2 comprises residues 736–751 from the gp41 region. Neither of these BDs was immunogenic in BALB/c mice. However, when covalently linked to vpr20 (constructs $TD_h$-BD1, $TD_h$-BD2, poly 1 and poly 2) high antibody titers were induced (Table 4). Polymerization enhanced the antibody titers against BD1 but lowered those against BD2. Although not explicitly shown, none of the

TABLE 1. *Synthetic overlapping peptides encompassing the sequence of vpr*

| Peptide | Sequence[a] |
|---|---|
| 01–15 (vpr7) | MEQAPEDQGPQREPH |
| 06–20 (vpr8) | EDQGPQREPHNEWTL |
| 11–25 (vpr9) | QREPHNEWTLELLEE |
| 16–30 (vpr10) | NEWTLELLEELKNEA |
| 21–35 (vpr11) | ELLEELKNEAVRHFP |
| 26–40 (vpr12) | LKNEAVRHFPRIWLH |
| 31–45 (vpr13) | VRHFPRIWLHGLGQH |
| 36–50 (vpr14) | RIWLHGLGQHIYETY |
| 41–55 (vpr15) | GLGQHIYETYGDIWA |
| 46–60 (vpr16) | IYETYGDIWAGVEAI |
| 51–65 (vpr17) | GDIWAGVEAIIRILQ |
| 56–70 (vpr18) | GVEAIIRILQQLLFI |
| 61–75 (vpr19) | IRILQQLLFIHFRIG |
| 66–80 (vpr20) | QLLFIHFRIGCRHSR |
| 76–90 (vpr22) | CRHSRIGVTQQRRAR |
| 81–95 (vpr23) | IGVTQQRRARNGASR |

[a] Peptides contain an extra Val at the C-terminus (not shown) that was added for convenience of synthesis.

TABLE 2. *Peptides from vpr recognized by HIV-1-seropositive patients with >500 $CD4^+$ cells/$mm^3$*[a]

| Patient | Recognized peptide |
|---|---|
| 8803 | vpr20 |
| 8813 | — |
| 8819 | — |
| A1 | vpr17, vpr20 |
| SDR | vpr10, vpr20 |
| JAT | — |
| AGF | vpr20 |
| FAS | vpr20 |
| NBM | — |
| CB | — |
| PJ | — |
| 8807 | — |
| 8810 | — |
| ACC | vpr20 |
| SG | — |
| DAE | — |

[a] The proliferation assay was carried out using 15 μg/ml of peptide. Only those peptides giving a stimulation index >3 are shown.

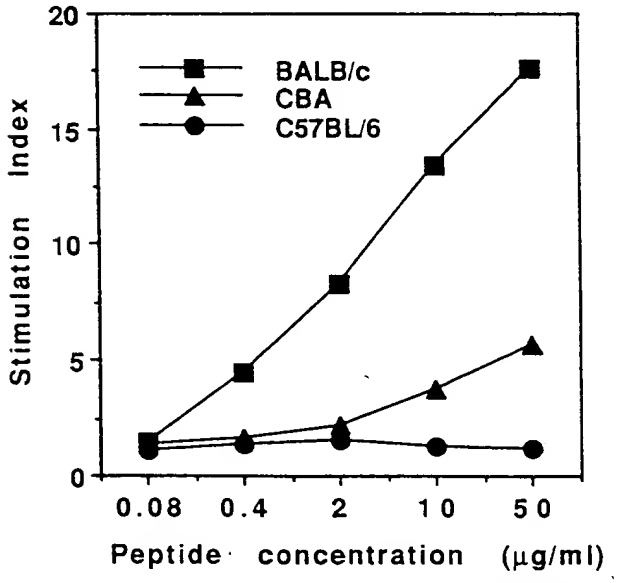


FIG. 1. Induction of IL-2 production in vpr20-immunized mice after in vitro restimulation of lymph node cells with vpr20.

TABLE 3. *Peptide constructs used as immunogens*[a]

| Peptide | Abbreviation |
|---|---|
| QLLFIHFRIGCRHSR | vpr20 or $TD_h$ |
| KSIRIQRGPGRAF | BD1 |
| RPEGIEEEGGERDRDR | BD2 |
| QLLFIHFRIGCRHSRKSIRIQRGPGRAF | $TD_h$-BD1 |
| QLLFIHFRIGCRHSRRPEGIEEEGGERDRDR | $TD_h$-BD2 |
| $(QLLFIHFRIGCRHSRKSIRIQRGPGRAF)_n$ | poly 1 |
| $(QLLFIHFRIGCRHSRRPEGIEEEGGERDRDR)_n$ | poly 2 |

[a] The $TD_h$ moiety is shown in bold.

constructs containing vpr20 induced antibodies against the vpr20 moiety.

### Neutralization of HIV-1 Infectivity by Anti-BD1 and Anti-BD2 Antisera

The inhibition (90% inhibition titer) of the infection of HT4LacZ-1 cells by HIV-$1_{IIIB}$ with the antisera induced with different peptide constructs is shown in Table 5. Clearly, the anti-BD1 antibodies induced with poly 1 are those induced by the constructs that have the highest inhibition titer. No neutralizing titers could be obtained with antibodies elicited against control peptides 1 and 2 from gp120.

### Specificity of Anti-BD1 Antibodies for Peptides from the Variant V3 Sequences

The specificity of anti-BD1 antibodies induced by $TD_h$-BD1 and poly 1 was tested by ELISA against a panel of 13 synthetic peptides (Table 6) corresponding to variant V3 loop sequences. Figure 2 shows that only peptides 4, 10, and 13 are recognized by anti-BD1 antibodies. Peptides 4, 10, and 13, but not the remaining peptides, share the amino acids QR of the central region of V3, which are characteristic of the HIV-$1_{IIIB}$ strain.

TABLE 4. *Antibody titers ($\times 10^{-3}$) at day 60 induced after immunization with the peptide constructs shown*[a]

| | Titrated against | |
|---|---|---|
| Immunized with | Free BD | Whole construct |
| BD1 | 0.3 | 0.3 |
| $TD_h$-BD1 | 10.6 | 300 |
| Poly 1 | 14 | 73 |
| BD2 | 0 | 0 |
| $TD_h$-BD2 | 21 | 46 |
| Poly 2 | 13 | 190 |

[a] Titers are the mean from the serum samples of three mice.

TABLE 5. *Inhibition of the infection of HT4LacZ-1 cells by HIV-1 with different anti-peptide antisera*

| Antibodies induced with | 90% Inhibition titer |
|---|---|
| $TD_h$-BD1 | 20 |
| Poly 1 | 80 |
| $TD_h$-BD2 | 10 |
| Poly 2 | 0 |
| Control peptide 1[a] | 0 |
| Control peptide 2 | 0 |

[a] Control peptides 1 and 2 correspond to sequences PTDPNPQEVVLVNVT and EQFGNNKTIIFKQSS from HIV-$1_{IIIB}$ gp120. They induced anti-peptide antibody titers of 66,000 and 58,000, respectively.

## DISCUSSION

Because memory T-helper cells play an essential role in the activation of B lymphocytes as well as of cytotoxic T lymphocytes (CTLs), vaccines against an infective agent should include one or more short peptide sequences, from the proteins of this agent, that after processing by the antigen-presenting cells (APCs) after natural infection are recognized as $TD_h$ by class II molecules of the host. Thus, the design of potential peptide vaccines implies the identification of these $TD_h$ sequences in infected individuals. These determinants have been used to potentiate the induction of neutralizing antibodies (16,17) and CTLs (18,19) against HIV-1.

The recognition of a BD by an immunoglobulin is not likely to be species-dependent. For this reason, the antibodies induced in an animal species may be used to predict the potential viral neutralization power of the corresponding immunoglobulin induced in another animal species. However, the recognition of a $TD_h$ sequence depends on the class II

TABLE 6. *Synthetic peptides from the V3 loop corresponding to sequences found in different virus isolates*

| Peptide | Sequence | Viral strain[a] |
|---|---|---|
| 1 | TRPNYNKRKRIHIGPG | MN |
| 2 | TRPNNNTRKSIRIGPG | Consensus C |
| 3 | TRPNNNTRKSIYIGPG | SF2 |
| 4 | PNNNTRKSIRIQRGPG | LAI |
| 5 | RKSIRIGPGRAFVTI | —[b] |
| 6 | RKRIHIGPGRAFYTT | MN |
| 7 | RKSIYIGPGRAFHTT | SF2 |
| 8 | RKSITKGPGRVIYAT | RF |
| 9 | HIGPGRAFYTIKNII | MN |
| 10 | RIQRGPGRAFVTIGK | LAI |
| 11 | YIGPGRAFHTTGRII | SF2 |
| 12 | TKGPGRVIVTIGQII | —[c] |
| 13 | KSIRIQRGPGRAF | LAI |

[a] As reported by Myers et al. (8).
[b] Peptide from LAI strain with QR deleted.
[c] Chimeric peptide from RF and LAI strains.

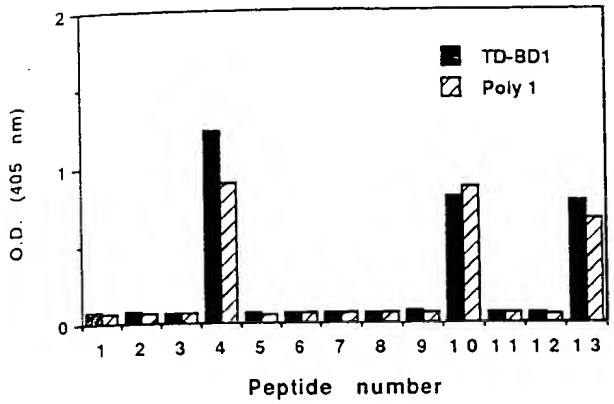


FIG. 2. Recognition of peptides from the V3 loop by 1:500 anti-($TD_h$-BD1) and anti-poly 1 antisera.

molecules of the individual. For these reasons, insight into the immunogenicity of peptide constructs $TD_h$-BD in humans can be obtained by immunization of mice with this type of construct, provided that the $TD_h$ moiety is recognized by man and mouse class II molecules. However, this strategy does not take into account differences in antigen processing among species.

In the context of HIV-1 infection, most efforts have been made to identify $TD_h$ sequences from its structural proteins (20–22). However, to our knowledge, no $TD_h$ from the regulatory protein vpr has yet been identified. Because this regulatory protein is highly conserved, any $TD_h$ from its sequence would have the advantage of being contained by most viral isolates, the sole restriction being associated with the class II molecules of the host. For this reason, we identified the $TD_h$ from vpr most commonly found in HIV-1-seropositive patients, with the purpose of engineering immunogenic peptide constructs to be tested in mice, but for potential use in humans. Table 2 shows that 37.5% of patients recognized peptide vpr20. This percentage of recognition might be related to the class II molecules of these patients. However, it cannot be excluded that the T cells of some of them were no longer able to proliferate in vitro under the conditions of the assay. Thus, the inclusion of this $TD_h$ in a peptide vaccine construct may provide T-cell help to an even wider population.

According to the principles discussed herein, the testing of constructs of the type vpr20-BD required an animal model in which vpr20 was recognized as a $TD_h$. Thus, we immunized three mice strains: BALB/c, CBA, and C57BL/6, which are, respectively, H-$2^d$, H-$2^k$, and H-$2^b$ restricted. From Fig. 1 it is clear that vpr20 is more immunogenic in BALB/c, and for this reason we chose this strain to test our peptide constructs.

We decided to use the peptides BD1 (from the V3 loop of gp120) and BD2 (from gp41) as BDs, because it has been reported that antibodies against these regions can neutralize HIV-1 infectivity (23,24). We chose the N-terminal position for the $TD_h$ moiety in the constructs based on previous work on the relative position of BD and $TD_h$ in the immunogens (4). Similarly, we prepared linear polymers of our peptide immunogens because polymerization may enhance the immunogenicity of this type of construct (12).

Table 4 confirms the finding (1–4) that the help from the $TD_h$ is essential to induce antibodies against a nonimmunogenic BD, BD1, and BD2. Although antibodies against the constructs were unable to recognize the $TD_h$ moiety, the titers against the whole constructs were higher than against the BD moieties. A higher specificity/affinity for the antigen used for immunization and/or a wider polyclonal antibody response might account for these differences in titer. The absence of anti-$TD_h$ antibodies after immunization with these constructs is desirable because it would avoid the neutralization of this moiety in successive immunizations.

Linear polymerization enhanced the antibody titers against the free BD1 moiety but lowered the titers against the whole $TD_h$-BD1 construct. An opposite effect was found for the case of the $TD_h$-BD2 construct. This finding shows that the effect of polymerization on peptide immunogenicity cannot be predicted and needs to be tested by a trial-and-error approach.

As shown in Table 5, the highest 90% inhibition titers are obtained with the antibodies against BD1, a result in agreement with the reported character of the principal neutralizing domain of loop V3, which contains BD1 (23). Although linear polymerization of $TD_h$-BD1 moderately enhanced the anti-BD1 titer, the neutralizing titer increased fourfold. This finding probably reflects the fact that the BD1 moiety in poly 1, but not in $TD_h$-BD1, is more structurally similar to the corresponding region on the surface of the virus. The specificity of these neutralizations was confirmed by the absence of neutralization when using antisera against control peptides 1 and 2.

As shown in Fig. 2, the antibodies against BD1 induced by either $TD_h$-BD1 or poly 1 were able to recognize the free BD1 (peptide 13) and also pep-

tides 4 and 10. However, they were unable to recognize the remaining peptides, showing that these antibodies are highly specific for the V3 region containing the sequence QR, which is found only in peptides 4, 10, and 13. This result suggests that to induce neutralizing antibodies against a wide range of viral strains, the immunogens should contain BDs from different V3 isolates (25) as well as from other neutralizing domains. Moreover, it would be advisable for these immunogens to contain several $TD_h$ from different regions of the viral proteins in order to cover differences in major histocompatibility restriction of the subjects and sequence variability due to mutation of HIV-1. Because vpr20 is highly conserved and recognized by a large proportion of infected individuals, we believe that this $TD_h$ is a promising candidate to consider in the design of future peptide vaccines.

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# Identification of HIV-1 *vpr* Product and Function

Eric A. Cohen, Ernest F. Terwilliger, Yassamin Jalinoos, Jeffrey Proulx, Joseph G. Sodroski, and William A. Haseltine

*Department of Human Retrovirology, Dana-Farber Cancer Institute, Harvard Medical School, Massachusetts U.S.A.*

**Summary:** To investigate the role of *vpr* (viral protein R) in the replication and cytopathicity of human immunodeficiency virus type 1 (HIV-1), infectious proviruses were constructed that were isogenic except for the ability to produce the protein product of *vpr*. The experiments described here demonstrate that *vpr* encodes a 96 amino acid 15 kDa protein. The *vpr* product increases the rate of replication and accelerates the cytopathic effect of the virus in T cells. *Vpr* acts in *trans* to increase levels of viral protein expression. The stimulatory effect of *vpr* is observed to act on the HIV-1 LTR as well as on several heterologous promoters. **Key Words:** Viral replication—Regulatory proteins.

Infection by the human immunodeficiency virus type 1 (HIV-1) may result in prolific, controlled or silent states of virus replication (1). Knowledge of factors that contribute to the complex states of natural infection is important to an understanding of disease progression and pathogenesis. Part of the explanation for the complex replication patterns of HIV-1 lies in the intricate genetic structure of the virus (2–6). In addition to *gag, pol,* and *env* typical of retroviruses, HIV-1 contains *tat, vif, vpu, rev,* and *nef* that modulate virus replication (Fig. 1) (6). The experiments described here demonstrate that HIV-1 *vpr* (viral protein R) open reading frame encodes a 96-amino acid 15 kDa protein. The *vpr* product increases the rate of replication and accelerates the cytopathic effect of the virus in T cells. *Vpr* acts in *trans* to accelerate viral protein expression. *vpr* is capable of stimulating in *trans* gene expression directed by the HIV-1 LTR as well as heterologous promoters.

The existence of a protein product of *vpr*, called R (7), was previously inferred by the conservation of an open reading frame in diverse HIV-1 isolates and by detection in some infected patients of antibodies that recognize peptides predicted to be made from this coding sequence (8). However, neither the protein product nor an activity of this genetic region has been reported heretofore.

Address correspondence and reprint requests to Dr. William A. Haseltine at Department of Human Retrovirology, Dana-Farber Cancer Institute, Harvard Medical School, Binney Street, Boston, MA 02115 U.S.A.

## RESULTS

### Characterization of the *Vpr* Product in Vitro

To determine whether the products made from the *R* region of diverse isolates correspond to the predicted molecular weights, an antiserum to a synthetic peptide corresponding to amino acids 1 to 19 of the R protein was made (Fig. 2). A set of plasmids was constructed in which the Sp6 bacteriophage RNA polymerase promoter (9) was placed immediately 5′ to the *R* regions derived from the IIIB (HXBc2 and BH10) (2), ELI (E-H12) (10), and BRU (pJ19-13) (3) proviruses. The segment of the proviruses inserted in these plasmids extends from the 5′ end of the *R* open reading frame through the beginning of *env* (Fig. 1). The genomic sequence of these strains predicts that the ELI and BRU proviruses make R proteins 96 amino acids long, whereas BH10 and HXBc2 proviruses (2) derived from IIIB

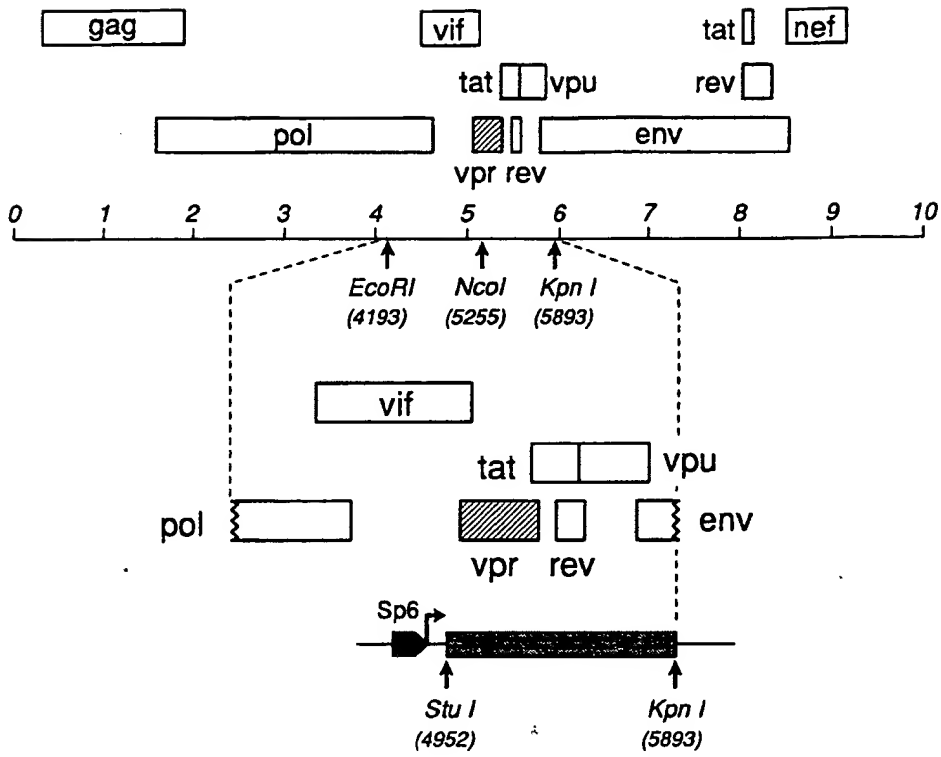



**FIG. 1.** HIV-1 genetic organization and plasmid constructs. Construction of HXBc2R$^+$ provirus. A segment of the HXBc2 provirus (ref. 2) located between the C terminal of *pol* (*Eco*RI site at nucleotide 4193, +1 = start of HXBc2 initiation of transcription) and the N terminal of *env* (*Kpn*I site at nucleotide 5893), a region that contains the entire *R* coding sequence (light shading), was replaced with the corresponding segment derived from the closely related BRU provirus (ref. 3) to yield the R$^+$ provirus designated HXBRU$^+$. A mutant of HXBRU$^+$ was constructed by digestion at the *Nco*I site (position 5255 +1 = start of BRU initiation of transcription) and insertion of four nucleotides with *Escherichia coli* DNA polymerase I Klenow fragment, causing a frameshift mutation after R residue 40, which truncates the protein and adds three amino acids. This otherwise isogenic provirus encoding an altered R was designated HXBRU$^-$. The alignment of the viral reading frame within the BRU fragment is shown. Transcription expressors encoding the region between the C-terminal of *vif* and the N-terminal portion of *env* derived from several proviruses were also made. Segments of HXBc2 BH10 and HXBRU (dark shading) were cloned into Sp6 transcription vector (ref. 9) using the conserved *Stu*I (position 4987) and *Kpn*I (position 5923) site indicated (the nucleotide positions indicated are for HXBc2). The fragment derived from the ELI provirus (E-H12) (ref. 10) spanned the region between a *Bam*H1 site at nucleotide position 5014 and a *Pst*I site at nucleotide position 5860.

make R protein 78 and 17 amino acids long, respectively (11) (Fig. 2). RNA prepared by in vitro transcription (9) of the plasmids was used to program a rabbit reticulocyte extract in the presence of [$^{35}$S]methionine (12).

Inspection of Fig. 3 reveals that the sizes of the proteins recognized by the anti-peptide sera differ. Proteins of 15 kDa were immunoprecipitated from reactions containing sequences of ELI and BRU, whereas the protein containing BH10 sequences was 8 kDa. The proteins recognized by the anti-R peptide serum were specifically competed for by the peptide itself. As predicted by the amino acid sequence, the antiserum failed to recognize the protein in the reaction that contained the HXBc2 *R* region sequence. The size of the proteins made in these reactions corresponds to that predicted from the nucleotide sequence (Fig. 2).

### Replication of Isogenic *vpr*-Expressing or *vpr*-Defective Proviruses

The observation that the proteins made by the *R* regions from independent proviral isolates differed in apparent molecular weight raised the possibility that the product of the *R* region of the BH10 clone was prematurely truncated because of an insertion of an additional thymidine between nucleotides 5351 and 5352 (+1 = site of initiation of transcription of BRU proviral clone), which changes the reading frame, and as a consequence that the function of the *R* region products of these strains might

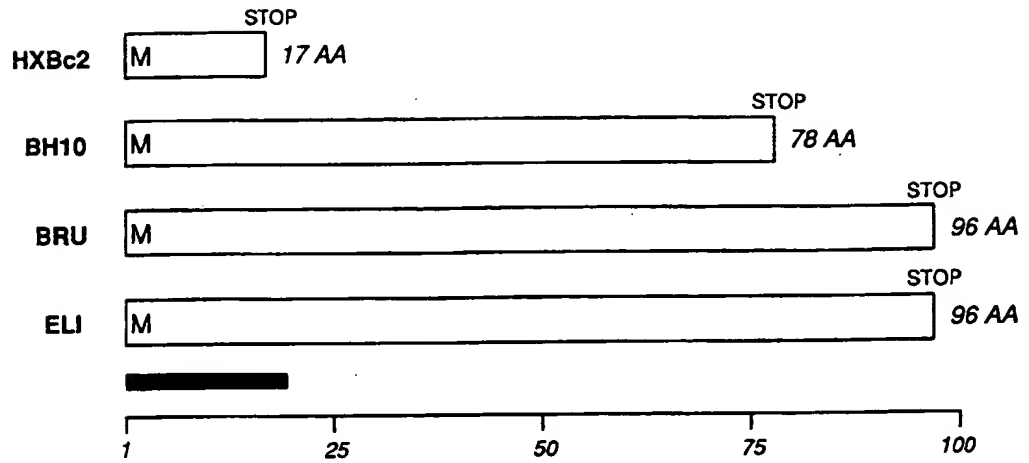



**FIG. 2.** Comparative analysis of the predicted *R* gene product from multiple HIV-1 provirus clones. The HIV-1 isolates compared are HXBc2, BH10 (ref. 2), BRU (ref. 3), and ELI (ref. 10). HXBc2 has a stop codon after amino acid 17. BH10, which was also isolated from the IIIB isolate, is predicted to make a protein of 78 amino acids. The ELI and BRU proviral clones are predicted to make a longer protein of 96 amino acids. The location of the nineteen amino-acid R synthetic peptide corresponding in sequence to *R* of the IIIB (BH10) sequence is also shown (dark bar). M indicates the position of the initiator methionine.

be defective. Previous attempts to define the activity of the *R* region have been made by introducing mutations into the 78 amino acid version of the *R* coding region contained within the BH10 and HXB2 (pX) subclone of IIIB (8,13). No effect of the open reading frame on virus replication was observed in these studies.

To test the possibility that the larger *R* region might specify an active protein, a pair of proviruses were constructed that differed only in the ability to produce the full length 15 kDa *R* product. The IIIB and BRU strains of HIV-1 are closely related in nucleotide sequence. A hybrid between HXBc2 and BRU proviruses was constructed in which the sequences between *pol* and *env* of HXBc2 were replaced by the corresponding sequences of BRU (Fig. 1). The genotype of the resultant virus was 5′ $LTR_{HXBc2}$ $gag^{+}{}_{HXBc2}$ $pol^{+}{}_{HXBc2}$ $vif^{+}{}_{BRU}$ $R^{+}{}_{BRU}$ $tat^{+}{}_{BRU}$ $rev^{+}{}_{BRU}$ $vpu^{+}{}_{BRU}$ $env^{+}{}_{HXBc2}$ $nef^{-}{}_{HXBc2}$ 3′ $LTR_{HXBc2}$ ($HXBRU^{+}$). An otherwise isogenic provirus was made that contained a frameshift mutation predicted to terminate the *R* product at amino acid 40 ($HXBRU^{-}$).

The role of the *R* region product in replication and cytopathic effect was assessed by transfection of a $CD4^{+}$ human T cell line (Jurkat) with equal amounts of the proviral DNA. Immunoprecipitation of labeled extracts of these cells with the anti-R peptide sera revealed the presence of a 15 KDa protein in lysates of cells exposed to $HXBRU^{+}$ DNA (Fig. 4). The 15 kDa protein has the same electro-

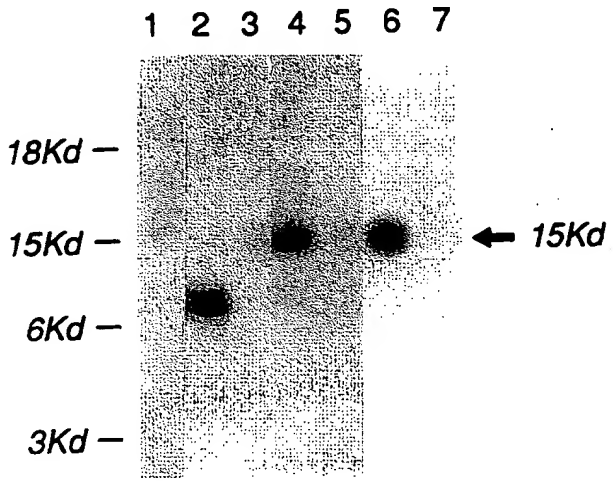



**FIG. 3.** In vitro characterization of *R* gene products from different proviruses. Transcription expressor plasmids encoding the *R* region derived from HXBc2 (ref. 2) (lane 1), BH10 (ref. 2) (lanes 2–3), ELI (ref. 10) (lanes 4–5), and $HXBRU^{+}$ (lanes 6–7) were linearized at a unique restriction endonuclease site located in the polylinker 3′ to the proviral fragment and used as template for in vitro transcription by Sp6 bacteriophage RNA polymerase (ref. 9). The methods used to produce the RNA and for in vitro translation and immunoprecipitation were those described previously (ref. 12). The RNA made in vitro has the capacity to encode *vpr*, the first exon of *tat* and *rev*, *vpu* in the case of BH10, ELI and BRU, and 40 amino acids of *env*. In vitro translation of equimolar amounts of RNA as determined by use of equal amounts of [$^{3}$H]cytidine 5′-triphosphate containing RNA, was performed in rabbit reticulocyte lysate. Incubation was at 30°C for 20 min in presence of [$^{35}$S]methionine. Labeled products were analyzed by immunoprecipitation with either anti-R peptide serum (lanes 1, 2, 4, 6) or anti-R peptide serum in the presence of 500 μ*M* of R peptide (lanes 3, 5, 7). Immunoprecipitations were analyzed on 12.5% SDS- PAGE.

phoretic mobility as the protein made in reticulocyte extracts and was specifically competed for by the peptide itself. No such protein was detected in lysates of cells transfected with the HXBRU$^-$ or HXBc2 DNA.

The cytopathic effects and replication rates of $R^+$ and $R^-$ virus differed by all measures (Fig. 5 and 6). Syncytia were evident by day 2 in cultures infected with $R^+$ virus, and the number of syncytia peaked by day 3 (Fig. 5A). Marked reduction in total cell number occurred by day 6 as compared to mock transfected control cultures (Fig. 5A). By contrast, the number of syncytia in cultures transfected with $R^-$ virus peaked 2 days later on day 5, and reduction in total cell number was not apparent until day 9. By day 3, abundant virus was detected in the medium of the cultures infected with $R^+$ virus, as measured by extracellular reverse transcriptase activity, and reached a high plateu level by day 6 (Fig. 5B). Virus production fell markedly by day 10, probably reflecting loss of viable cells. Virus production was not detected in cultures infected with

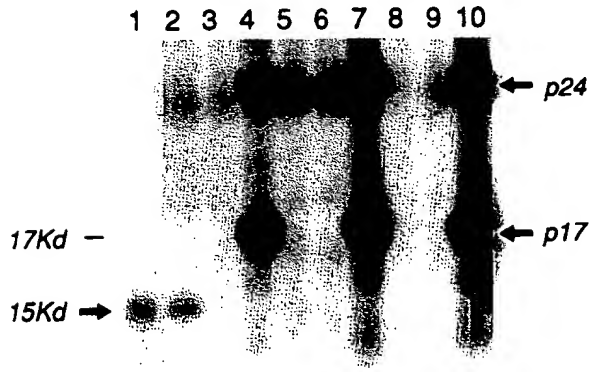
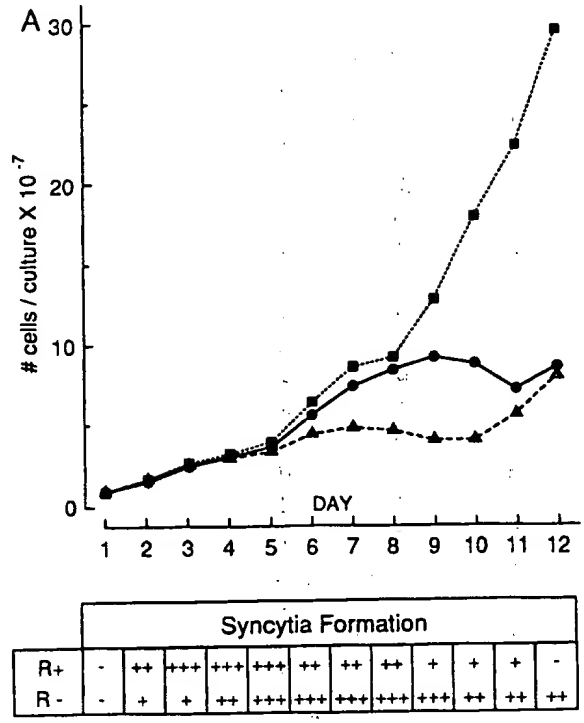



FIG. 4. Detection of R protein in infected Jurkat cells. $10^7$ cells were transfected with 10 μg of either HXBRU$^+$, HXBRU$^-$, or HXBc2 using a DEAE-dextran transfection technique (ref. 14). Six days post-transfection cells that were metabolically labeled with 100 μCi/ml [$^{35}$S]cysteine and 200 μCi/ml [$^3$H]arginine were collected and immunoprecipitated as described (ref. 24); HXBRU$^+$ infected Jurkat cells were immunoprecipitated with anti-R peptide serum (land 2), anti-R peptide serum following a 1 h preincubation of the serum with 500 μ*M* of R peptide (lane 3), or AIDS patient antiserum (lane 4); HXBRU$^-$ infected cells immunoprecipitated with anti-R peptide serum (lane 5), R peptide antiserum plus peptide (lane 6) or AIDS patient antiserum (lane 7); HXBc2 infected cells immunoprecipitated with R peptide antiserum (lane 8), R peptide antiserum plus peptide (lane 9), or AIDS patient antiserum (lane 10). Lane 1 represents the immunoprecipitation of the BRU R protein synthesized in vitro in a rabbit reticulocyte lysate and immunoprecipitated with the anti R peptide serum. Lane 1 was run on the same gel with a shorter exposure.



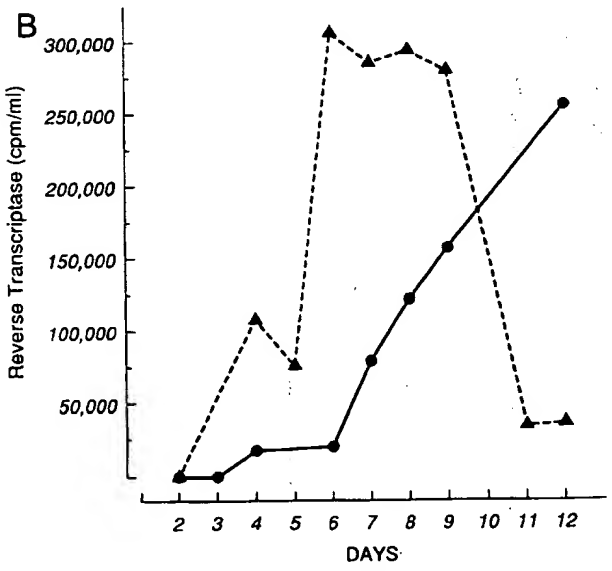




FIG. 5. Replication studies of $R^+$ and $R^-$ viruses within CD4$^+$ lymphocytes. $10^7$ Jurkat cells were transfected on day 0 with 10 μg of either HXBRU$^+$ or HXBRU$^-$ as described (ref. 14). Afterward, the cells were suspended in 15 ml of RPMI + 15% fetal bovine serum. All transfections were subsequently given a complete medium change daily. Effects of virus infection within each culture were then monitored at regular intervals for **(A)** cell number and syncytia formation and **(B)** supernatant reverse transcriptase activity as described (ref. 25). (---▲---) HXBRU$^+$, (—●—) HXBRU$^-$, (---■---) mock-infected cells.

$R^-$ virus until day 7 and continued to increase through day 12 (Fig. 5B).

The cultures were labeled with [$^{35}$S]cysteine on days 3–4, 6–7, 9–10, and 12–13. Lysates of cells were immunoprecipitated using AIDS patient antiserum (Fig. 6). Viral proteins were detectable in the lysates of cultures transfected with $R^+$ virus by day 3 post-transfection, peaked between day 6 to day 9, and declined by days 12–13. By contrast, cell-associated viral proteins in cells infected with $R^-$ virus first appear between days 6 and 7 and persist at a high level through days 12–13, reflecting continued viability of this culture. Similar results were obtained in three independent experiments. The results obtained using similar amounts of $R^+$ and $R^-$ virus (as determined by p24 concentration and reverse transcriptase activity) to infect Jurkat cells also revealed differences in the rate of replication and cytopathic effect similar to those obtained in the transfection experiments (data not shown).

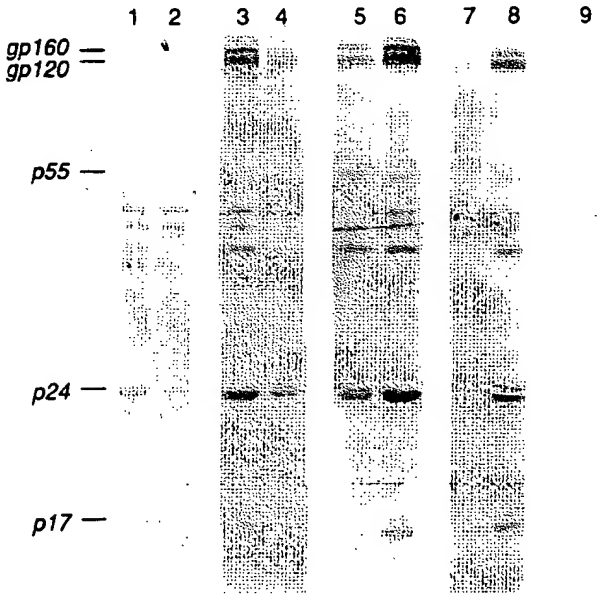



**FIG. 6.** Immunoprecipitation of serially labeled infected Jurkat cell lysates with AIDS patient antiserum. Five ml aliquots of infected Jurkat cells were pelleted, washed once with PBS, and resuspended in 3.0 ml of cysteine-free RPMI supplemented with 10% fetal bovine serum plus 50 μCi/ml of [$^{35}$S]cysteine. Cells were labeled overnight on day 3 (lane 1–2), 6 (lane 3–4), 9 (lane 5–6), and 12 (lane 7–8) post-transfection, then collected and immunoprecipitated with AIDS patient antiserum as described (ref. 25). Proteins were separated by 12.5% SDS–PAGE. Lanes 1, 3, 5, and 7: HXBRU$^+$-infected cells; lanes 2, 4, 6, and 8: HXBRU$^-$ infected cells; lane 9: mock-infected Jurkat cells. A similar lag in the expression of viral proteins associated with virus particles in the cell supernatant was observed between HXBRU$^+$ and HXBRU$^-$ infected cultures.

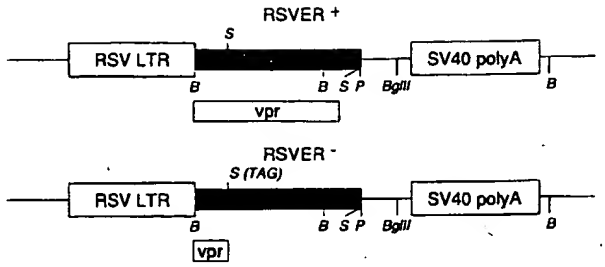



**FIG. 7.** *R* expressors plasmids. Plamid RSVER$^+$ was constructed by inserting a *Bam*H1 (5014) to *Sty*I (5428) fragment from the ELI provirus (ref. 10) 3′ to the Rous sarcoma virus (RSV) long terminal repeat (LTR) and 5′ to an SV40 16S RNA splice and poly (A) sites (ref. 15). The 414 base pair viral fragment extends from the 3′ end of *vif* to 53 nucleotides within the *tat* sequence and has only the capacity to encode the 96 amino acids R protein. The control plasmid, RSVER$^-$, was produced by introducing a *Nhe*I linker at an internal *Sty*I site (5208) creating a premature termination codon after R residue 35. RSVERΔ3 was constructed by inserting a *Bam*H1, fragment (5014–5379) from the ELI provirus 3′ to the RSV LTR and 5′ to the SV40 16S RNA splice and poly (A) sites (ref. 15). The 365 base pair viral fragment encode a shorter R protein of 93 amino acids. The BRU *R* expressor plasmids were constructed by cloning a *Stu*I (4987) to *Kpn*I (5923) fragment from HXBRU$^+$ and HXBRU$^-$ 3′ to the RSV LTR and 5′ to an SV40 16S RNA splice and poly (A) sites. The open boxes represent RSV sequences, the darker shaded boxes HIV-1 sequences, the lightly shaded boxes SV40 sequences, B = *Bam*H1; S = *Sty*I; P = *Pst*I.

### *Trans*-Activating Effects of *vpr*

To determine whether the product of the *R* region could act in *trans*, an *R* expressor plasmid was cotransfected with an indicator HIV-1 provirus that is defective for *R* expression (HXB-CAT) (14). The *R* region of HXB-CAT provirus contains a premature termination codon at amino acid 17 (2). The gene for the bacterial chloramphenicol acetyltransferase (CAT) replaces *nef* in HXB-CAT and is expressed in cells transfected with this provirus (14). The *R* expressor plasmid (RSVER$^+$) contains the *R* open reading frame of ELI located 3′ to the promoter of the Rous sarcoma virus (RSV) (nucleotides −491 to +33) (15) (Fig. 7). As a control, a premature termination condon was introduced within the *R* region of RSVER$^+$ at amino acid 35 to yield plasmid RSVER$^-$ (Fig. 7).

The ability of the *R* region to activate expression from the HXB-CAT provirus was determined by measuring the level of the viral capsid antigen, p24, in cell supernatants and the level of CAT activity in the transfected cells. Table 1 shows that the amount of p24 exported into the culture media and CAT activity were increased almost two to threefold in the presence of a functional *R* gene as compared to

TABLE 1. *Transactivation of viral protein expression by the* R *gene product*

| | 24 h | | 48 h | | 72 h | |
|---|---|---|---|---|---|---|
| | % CAT/min | ng p24/ml | % CAT/min | ng p24/ml | % CAT/min | ng p24/ml |
| RSVER$^+$ | <0.1 | 4.8 | 0.6 | 7.2 | 6.0 | 9.9 |
| RSVER$^-$ | <0.1 | 3.2 | 0.2 | 3.8 | 1.9 | 4.8 |

Ten micrograms of HXB-CAT provirus (ref. 14) DNA were cotransfected with 5 μg of either RSVER$^+$ or RSVER$^-$ in $10^7$ Jurkat cells. CAT activity and p24 levels were measured at 24, 48 and 72 h post-transfection as described (ref. 14). CAT activity is expressed as percent conversion of [$^{14}$C]chloramphenicol per min by 70 μl of 1:10 dilution of the original sample. Extracellular p24 level were assayed with commercial HIV p24 radiommune assay kit according to manufacturer directions.

the amount made upon transfection with the *R* negative control.

The capacity of the *R* expressor plasmid to increase the amount of viral proteins and CAT activity for the HXB-CAT provirus suggests that the *R* product might act in *trans* to stimulate HIV-1 LTR-directed gene expression. Accordingly, the ability of the *R* expressor to stimulate HIV-1 LTR-directed CAT expression (16) was tested in Jurkat cells. The data of Fig. 8 show that both the BRU and ELI *R* products activate the HIV-1 LTR-derived–directed gene expression in *trans* by threefold. The threefold stimulation of the HIV-1 LTR-directed CAT expression was also observed in the presence of an active *tat* product (data not shown). It is noteworthy that the *R* product also increases the level of CAT activity directed by heterologous viral transcription indicator sequences (Table 2). The *R* product was able to stimulate the SL3 LTR and RSV-directed CAT activity (15,17) eight- to tenfold (Fig. 9 and Table 2) as well as the human T-cell leukemia virus type I (HTLV-I), cytomegalovirus (CMV), and simian virus 40 (SV40) promoters, threefold (Table 2). The stimulation of both the HIV-1 and SL3 LTR CAT expression was dependent on the amount of transfected *R* expressor plasmid in the range of 0.1 to 2 μg of DNA (Fig. 8 and 9).

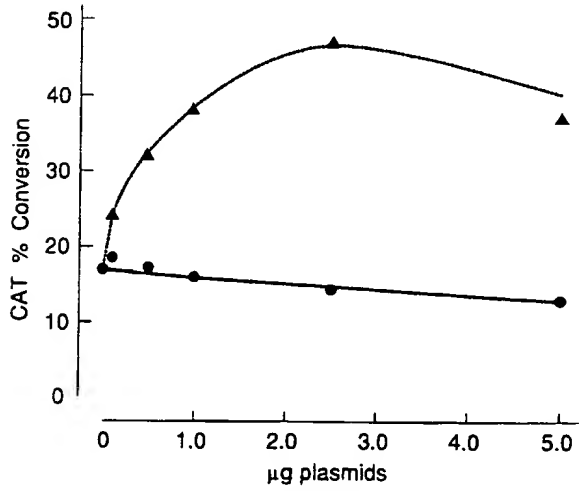



FIG. 8. Effect of ELI *R* product on HIV-1 LTR. Indicated amount of RSVER$^+$ or RSVER$^-$ were cotransfected with 10 μg of pU3RIII (ref. 16) in $10^7$ Jurkat cells as described (ref. 14). Seventy-two hours post-transfection CAT assays were performed as described (ref. 26) with 1 h incubation. All values were calculated from at least two independent transfections. The converstion rate (%) were determined from the linear portion of the assay using identical amounts of proteins (•••▲•••) RSVER$^+$; (—●—) RSVER$^-$. Similar dose–response curves were obtained using BRU *R* expressor plasmids.

TABLE 2. *Stimulation of heterologous enhancer promoter directed gene expression by the* R *gene product*

| Enhancer-promoter | RSVER$^-$ | RSVER$^+$ | RSVERΔ3 |
|---|---|---|---|
| HIV-1 | 1 | 3 ± 0.05 | 1.6 ± 0.2 |
| p-167 | 1 | 3 ± 0.5 | ND |
| p-57 | 1 | 3 ± 0.5 | ND |
| p-167/-17 | 1 | 3 ± 0.5 | ND |
| HIV-2 | 1 | 3 ± 0.5 | ND |
| SL3 | 1 | 10 ± 2.0 | 4 ± 0.5 |
| RSV | 1 | 8 ± 1.0 | 4 ± 0.5 |
| HTLV-I | 1 | 3 ± 0.5 | ND |
| CMV | 1 | 3 ± 0.5 | ND |
| SV40 | 1 | 3 ± 0.5 | ND |

2.5 μg of either RSVER$^+$ or RSVER$^-$ were cotransfected with the following enhancer promoter CAT constructs; 10 μg HIV-1 LTR-CAT, pU3RIII (−456/+80, +1 = site of initiation of transcription) (ref. 16); 10 μg of the following HIV-1 LTR deletion CAT constructs (ref. 18) p-167 (−167/+80), p-57 (−57/+80), p-167/−17 (−167/−17); 10 μg HIV-2 LTR-CAT, −556/+156 (ref. 27); 5 μg RSV LTR-CAT, pRSVCAT (−491/+33) (ref. 15); 5 μg HTLV-I LTR-CAT, pU3RI (−350/+315) (ref. 28); 2 μg of SL3 LTR CAT; pSU3CAT (−441/+33) (ref. 17); 2 μg CMV immediate early promoter-regulatory region-CAT, pTJ278 (−990/+66) (ref. 29); 2 μg SV40 early promoter CAT, pSV2CAT (ref. 26) in $10^7$ Jurkat cells except for pTJ278 and pSV2CAT where transfection were performed in $10^6$ HeLa cells as described (ref. 30). Seventy-two hours post-transfection CAT activity was measured as described (ref. 26). CAT activity is normalized to the activity obtained with the control *R* expressor plasmid, RSVER$^-$. Results represent the average of at least two independent transfections. The actual percentages CAT conversion for pU3RIII, p-167, p-57, and p-167/−17 in the presence of RSVER$^-$, in one experiment, were, respectively, 6.1, 6.4, 1.0, and 8.1%. When RSVER$^+$ was cotransfected, the percentages CAT conversions were 17.9, 18.6, 2.9 and 23%. ND: not determined.

*Trans*-activation of the HIV-1 LTR CAT expression provides an opportunity to examine the activity of variant *R* region products. Removal of the three carboxy terminal amino acids from the ELI *R* region product substantially reduced the activity of the gene (Table 2). The defective nature of the BH10 and HXB2 region products probably accounts for the earlier reports (8,13) that mutations in the *R* region do not affect virus replication as the *R* region of these strains appears to be defective to start with.

A series of mutant HIV-1 LTR sequences were used in an attempt to determine the location of sequences responsive to the *trans*-activating effect of the *R* product. However, as long as the mutation in the transcriptional element permitted detection of CAT activity, a significant increase in CAT expression was observed in the presence of an active *R* product. Deletion of HIV-1 enhancer (plasmid p-57), *tat* responsive sequences (TAR) (plasmid p-167/-17) and the negative regulatory element (NRE) (plasmid p-167) (18) did not affect *trans*-activation by the R product (Table 2).

## DISCUSSION

These results identify the product of *vpr* of HIV-1 as a 15 kDa protein. The *vpr* product acts to accelerate the cytopathic effect of HIV-1 in CD4$^+$ T cells and accelerates viral protein production and replication.

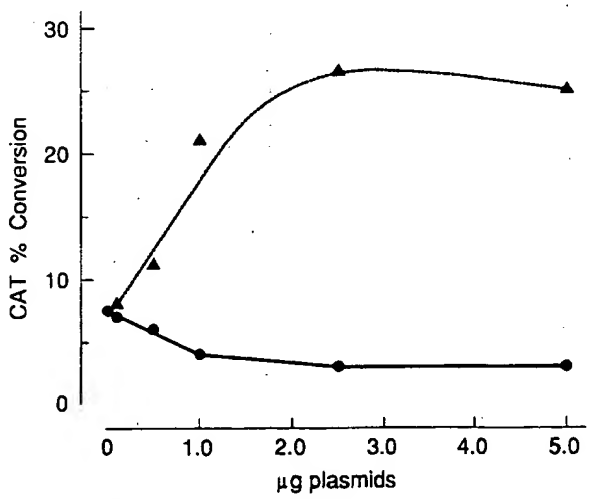



**FIG. 9.** Effect of ELI R product on SL3 LTR. Increasing amounts of RSVER$^+$ (•••▲•••) or RSVER$^-$ (—●—) were cotransfected with 2 µg of SU3CAT plasmids (17) in $10^7$ Jurkat cells. Seventy-two hours post-transfection, cells were lysed and CAT assays were performed on identical amounts of protein as described (ref. 26) with 30-min incubations.

Acceleration of viral proteins expression by *vpr* is dramatic. Approximately 10 times the level of HIV-1 viral proteins are made by days 3–4 postinfection with *vpr*$^+$ as compared to *vpr*$^-$ virus. The acceleration of cytopathic effects is also dramatic. Reduced cytopathic effects of *vpr*$^-$ virus may account for in vitro selection of *vpr*$^-$ mutant viruses. The *vpr* product acts in *trans* to increase the expression of viral proteins from the HIV-1 provirus. The transactivation is two- to threefold. This relatively small effect may not account for the significant change in viral protein production and cytopathic effects observed with *vpr*$^+$ provirus. The 15-kDa *vpr* protein also acts in *trans* to increase the level of expression of CAT directed by the HIV-1 LTR as well as other promoters. Transactivation by *vpr* is distinct from that of the two other virally encoded tranactivators *tat* and *rev*, as neither the *tat* responsive region (TAR) (18) nor the *rev* responsive region (CAR) (19,20) is required for *vpr* tranactivation.

The entire *R* open reading frame appears to be essential for activity of the *vpr* product, as the activity of a mutation that removes the three carboxy terminal amino acids is substantially reduced. No specific sequence in the HIV-1 LTR has been shown to be responsive to *vpr*. In this respect, *vpr* resembles several other viral *trans*-activator proteins, such as E1A (21), which stimulate a wide spectrum of viral and cellular promoters and for which it is difficult to pinpoint a responsive element. The existence of a transactivator gene of HIV-1 that is capable of altering heterologous gene expression function raises the possibility that *vpr* may play a role in pathogenesis by altering the expression of cellular genes.

The *vpr* product is dispensable for virus replication, as viruses incapable of expressing a functional *vpr* protein replicate in culture. Nonetheless, the *vpr* function appears to play an important role in natural infections, as some patients make detectable levels of antibodies to the *vpr* protein (8), and the open reading frame is present in HIV-1 (11) as well as in HIV-2 strains (22). A gene corresponding to *vpr* is also present in most strains of simian immunodeficiency virus (SIV) (23).

The *vpr* product is made from a partially spliced messenger RNA. Accumulation of such RNAs is dependent upon *rev* function and occurs late in the replication cycle at a time when expression of a *tat* protein may be declining. The additional boost in viral protein synthesis provided by *vpr* may help to ensure abundant production of virus particles late in the infection cycle.

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Note added in proof: While this manuscript was under review a paper by Ogawa et al. (*J Virol* 1989;63:4110–4) was published which is in agreement with the data showing increased growth rate of *vpr*$^+$ as compared to *vpr*$^-$ viruses (Figs. 5 and 6).

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